STUDIES ON IMPORTANT PHYTOCHEMICALS AND GENETIC TRANSFORMATION OF THE CYANOBACTERIUM *SPIRULINA*

A Thesis submitted to the
University of Mysore

for the award of
Doctor of Philosophy
in Biochemistry

by

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DECLARATION

I hereby declare that this thesis entitled **"Studies on important phytochemicals and genetic transformation of the cyanobacterium Spirulina"** which is submitted to the University of Mysore for the award of the degree of " Doctor of Philosophy' in Biochemistry, is the result of work carried out by me in Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore-570 013, under the guidance of Dr. T.N. Prabha during the period 1992-1996.

I further declare that the results of this work are not previously submitted for any degree or fellowship.

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CERTIFICATE

I hereby certify that the thesis entitled **"Studies on important phytochemicals and genetic transformation of the cyanobacterium Spirulina"**

which is submitted by Miss KM. Priya sethu to the University of Mysore for the award of the degree of ' Doctor of Philosophy' is the result of the work carried out by her in Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore-570 013, under my

T.N. PRABHA
Research Supervisor

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Mysore . **KM. Priya sethu**
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CONTENTS

LIST OF ABBREVIATIONS

A	Absorbance
A.E.	Antioxidative Effect
Amp	Ampicillin
APC	Allophycocyanin
ca,	approximately
cm	centimetre
d	day
Da	Dalton
DNA	Deoxy ribonucleic acid
DW	dry weight
FAa	Food and Agriculture Organization
FTCC	Food Technology Culture Collection
GLA	Gamma linolenic acid
HPLC	High Pressure Liquid Chromatography
hr	hour
g	gram
Kd	Kilo dalton
kg	kilo gram
Klux	Kilo lux
L	Litre
m	metre
mg	llll.. gram
min	minute
ml	milli litre
mm	milli metre
Mol. wt.	Molecular weight
O.D.	Optical Density
PBS	Phycobilisome
PC	Phycocyanin
RDA	Recommended Dailv Allowance
RNA	Ribonucleic acid
Rs.	Rupees
sec	second
SD	Standard Deviation
SOD	Superoxide dismutase
Tet	Tetracycline
TLC	Thin Layer Chromatography
U.S.	United States
UV	Ultraviolet
v, vol	volume
vito	vitamin
w, wt	weight
W	watt
µ	micron
µE	micro Einstein
µg	micro gram
µl	micro litre
$	dollar
°C	degree centigrade

LITERATURE REVIEW MICROALGAL BIOTECHNOLOGY: A SPECIAL REFERENCE TO SPIRULINA

CONTENTS

The need to feed and provide for a rapidly growing world population and the dwindling of many natural resources have placed present day, agriculture at crossroads. About three-quarters of all fertile land in the temperate and tropic zones is now devoted to agriculture. Science-based rather than resource-based agriculture is considered important for obtaining high productivity. This is achievable by prudent utilization of unique environments which would support Earth's natural ecosystems. In this context, large-scale microalgal culture represents an interesting possibility.

MICRO ALGAE: IMMORTAL LIFE FORMS

Algae represent two-thirds of the Earth's plant biomass [1]. Thousands of these algal species covering the Earth are now being identified and developed for food, Pharmaceuticals, biochemicals and fertilizers. As human food, microalgae have appeared at several interesting junctures in human history. They range in size from a single cell to a giant kelp of nearly 150 feet long. Most algae live depending on sunlight through photosynthesis, but some depend on organic matter.

Microalgae in the ocean, called phytoplankton form the base of the food chain and support all higher life. The rich upwellings of nutrients caused by the major currents from the sea, or nutrients from major river basins sustain phytoplankton growth. There are blue-green microalgae like Spirulina and Anabaena, green algae like Chlorella and Scenedesmus, red algae like Dunaliella, and also brown, purple, pink, yellow and black microalgae. They are everywhere-in water, in soils, on rocks and on plants. Blue-green algae are the most primitive organisms, and contain no nucleus or chloroplast. Their cell walls are composed of mucopolysaccharides responsible for the soft, slimy nature of the organisms.

MICROALGAL CULTIVATION

AN EVOLUTIONARY STEP IN AGRICULTURE

Modern agriculture has boosted short term productivity which is achieved by simply ignoring many hidden costs, such as the consumption of non-renewable fossil fuels for fertilizers and machinery, pollution of soil and water through excessive use of chemical fertilizers, and depletion of soils. This has led to tremendous ecological damage.

Ecological food production is the next stage in agriculture. This represents both an increase in productivity and protection of the Earth's resources. Organic and biodynamic farming methods, aquaculture and low tillage farming are practices now becoming more popular. Algae cultivation is a new addition to ecological food production. Soybean, a recent newcomer in protein production, took 50 years to emerge from obscurity. The rapid progress in microalgal technology for ' food/feed' and specific ' products' in the last two decades has been astounding.

ADVANTAGES

Microalgae have scarcely been commercially exploited, in sharp contrast to macroalgae ("seaweeds") which are used commercially for their polysaccharides like agar, alginic acid and carrageenan. Thus microalgae, comprising at least some 30,000 species [2], represent in effect an untapped natural resource. The many advantages of producing microalgae for economic purposes are:

* The microalgae are the most efficient biological systems for harvesting solar energy and for production of organic compounds via the photosynthetic process.

* The entire biomass is available for harvest and use, as most algae are non-vascular plants and lack complex reproductive organs.

* Many species of algae can be induced to produce high concentrations of chosen compounds, viz. proteins, carbohydrates, lipids, glycerol and pigments that are of commerical value [3, *4]*. Of particular interest is increased productivity, yielding a basic protein supply to regions of low productivity.

* Microalgal cultivation doesn't require prime agricultural land and so do not compete with it. Waste lands, hill slides and coastal zones, with a little engineering offer fine sites for algal cultivation.

* Genetic selection and strain screening are relatively easy and quick as these microorganisms undergo a simple cell division with a very short life cycle. This allows a more rapid development and demonstration of viable production processes than other agricultural processes.

* Microalgae can be grown using marginal (brackish or sea) water and some simple nutrients like *CO2* from photosynthesis and N2 from nitrogen fixation.

* Algal biomass production systems can be easily adapted to various levels of operational skills and investment, from simple, labour intensive production units to fully automated, capital intensive operations [5].

REQUIREMENTS

The process of producing microalga is presented in **Figure 1,** illustrating the basic inputs and potential uses of algal biomass. It is clear that algal biotechnology gives highly potential outputs from simple basic inputs. Shifrin [6] has made a comparative assessment of algal culture with agriculture in general terms. His observation is reproduced in **Table 1.**

WORLDWIDE POPULARITY

In Japan and Taiwan, a number of commercial microalgal production plants have operated for about 20 years, and several new plants were established in

Figure I. Basic inputs and potential uses of algal biomass

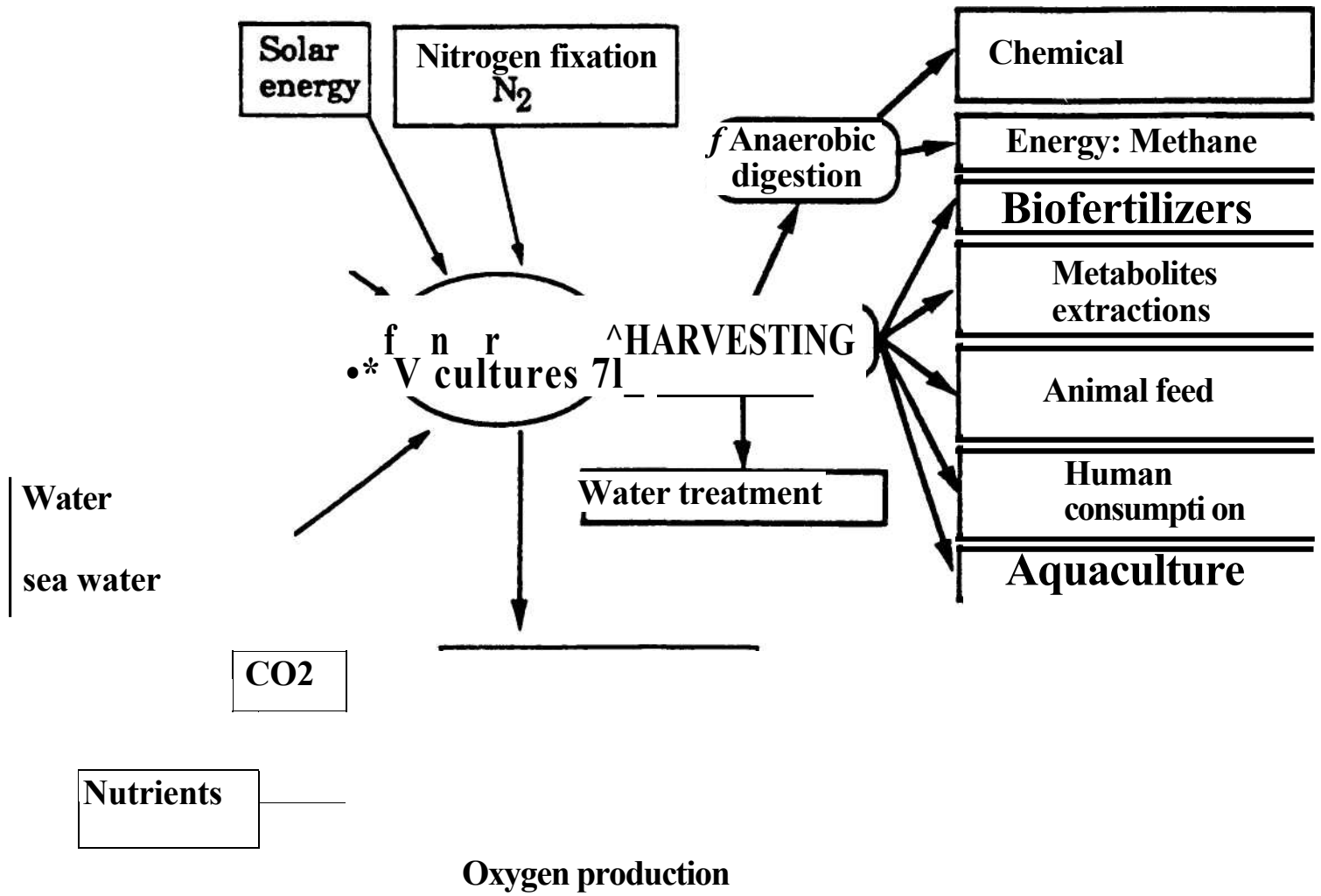

Table 1 : Agriculture vs algal culture

	Agriculture	**Algal culture**
Commercial acceptance	Established	R & D
Yields/Unit area	Low	High
Harvesting technology	Established	Developing
Residue waste Land	High	Low
requirement	High quality land	Marginal land
Water:		
Quality	Fresh water	Fresh, brackish
Quantity	Evaporation, run off	Evaporation
Genetic optimization	Mostly achieved	As yet untapped

Data from
Shifrin, N.S. 1984. Biotechnology for the Oils and Fats Industry. In: C. Ratledge, P. Dawson and J. Rattray (eds), American Oil Chemists' Society, Ilinois.

U.S. and other countries recently for natural pigments, vitamin supplements and health foods. Microalgal cultures are also used in waste-water treatment and in aquaculture for production of shell fish. In US and Europe, major R&D programmes have been underway for over two decades to develop algal biomass production as a future source of fuels [7].

COMMERCIAL APPLICATIONS

Microalgae biotechnology is rapidly moving from laboratory R&D and pilot plant operations to commercial reality. Significant technical problems remain to be solved before microalgae biotechnology is fully exploited for potential. products and processes. However, the progress made in the past decade has firmly established microalgae as part of the repertoire of industrial microbiologists. **Table 2** presents some of the algae currently grown on a large scale with their applications. A brief overview of the' products from microalgae is presented in **Table 3** [8].

CYANOBACTERIA

THE BLUE-GREEN ALGA : ITS POTENTIALS

In recent years, these algae have been receiving world-wide attention, paving the way for advanced biotechnology of cyanobacteria. They can grow in diverse and extreme environments. They are often partners in symbiotic relationships and have a wide-range of hosts [9]. The ability of cyanobacteria to withstand nutritional deprivation and other environmental stresses has made it possible to employ them in desert reclamation [10], waste-water treatment [11] and bioremediation [12]. Further, the isotopic labelling of cyanobacterial components using inexpensive substrates such as $2H_2O$ and 13CO2 [13, 14] is also possible due to their simple nutritional requirements.

Cyanobacteria could be used to deliver toxin to grazing mosquito larvae [15]. All the dominant Cyanobacteria in rice field ecosystems are N2 fixing

Table 2 : Microalgae of commercial interest grown under outdoor conditions

Algae name	Product	Use of market
Spirulina	Protein, pigments	Health food, feed, aquaculture
Dunaliella	B-carotene	Health food, feed
Porphyridium	Polysaccharides, pigments	Soil conditioning, food industry, diagnostics
Chlorella	Protein	Health food
Chlamydomonas	Polysaccharides	Soil conditioning
Anabaena	Nitrogen compounds	Biofertilizers
Isochrsis & Tetraselmis	Fatty acids	Aquaculture

Table 3 : Products from microalgae

Table 3 : Products from microalgae

Algal products	Uses	Product value	Market size	Algal source	Product content	Growth systems	Status
Isotopic compounds	diagnostics, research	> $1000/kg	small	many	1–10%	enclosed reactors	commercial
Phycobili-proteins	diagnostics:	> $10000/kg	small	red algae and	1–5%	enclosed	commercial
	food color:	> $100/kg	small	blue-greens		reactors	commercial
Pharmaceuticals	anticancer, antibiotics	high	large	blue-greens other?	0.1–1%	enclosed reactors	research
Beta-carotene	pro-vitamin A, food color	> $500/kg $300/kg	medium large	*Dunaliella*	5%	lined pond	commercial
Xanthophylls	chicken feeds:	$200–500/kg	medium	greens, others	0.2–0.5%	unlined	research
	salmon feeds:	> $1000/kg	small	*Haematococcus*	> 1%	pond	research
Vitamins (C & E)	natural vitamins	C: > $10/kg	medium	greens,	< 1 ?	fermentor	research
		E: > $50/kg	medium	others?		fermentor	research
Health foods	food supplements	$10–20/kg	medium to large	*Chlorella* *Spirulina*	100%	lined pond	commercial
Ion exchangers	heavy metal biosorption	$5–50/kg	large	various	10–20%	lined pond	commercial
Polysaccharides	viscosifiers, gums	$5–10/kg	medium to large	red algae others?	50%	lined pond	research
Bivalve feeds	seed production	$20–100/kg	small	diatoms, chrysophytes	100%	lined pond	commercial
Soil inoculum	conditioners:	$ > 100/kg	unknown	greens	100%	indoor	commercial
	fertilizers:		unknown	blue-greens		lined pond	research
Amino acids	proline:	$5–50/kg	small	*Chlorella*	10%	lined pond	research
	arginine:	$50–100/kg	small	blue-greens	10%	lined pond	conceptual
	aspartate:	$2–5/kg large	small		10%	lined pond	conceptual
Marine oils	food supplements	$1–30/kg	small	diatoms, others?	15–30%	lined pond	research
Waste treatment	general waste waters	$1–5/kg algae biomass	large	greens others?	n.a.	unlined pond	commercial
Liquid fuels	Substitute for oil	$0.1–$0.2/kg	large	greens, diatoms	30–50%	unlined pond	research

Not all potential microalgae products are listed. Product values indicate only general price estimate. Market sizes ($ million): small < $10; medium $10–100; large > $100.

Estimated content as % of dry weight of biomass; n.a.: not applicable.

Growth systems are the most likely bioreactor used in production of the microalgae biomass.

Data from

Benemann, J.R. 1990. Microalgae products and production: an overview. Developments Industr. Microbiol. **31:** 247-256.

[16]. There is an extensive literature concerning attempts to use either free-living or symbiotic cyanobacteria for agricultural advantage [17]. Apart from their primary products, they also produce secondary metabolites, which are potential pharmaceuticals [18]. Phycobiliproteins (phycocyanin, phycoerythrin) which are intensely fluorescent proteins with a broad range of spectral characteristics [19, 20] and a large variety of carotenoids have a very wide commercial application [21]. C-phycocyanin is already used in cosmetics [Dainippon Ink and Chemicals Inc. 1981] and marketed as research diagnostics. In bioreactors, immobilized cells of certain cyanobacteria may provide the mcans for the production of ammonia on an industrial scale [22].

Cyanobacteria (apart from dinoflagellates) are major sources of neurotoxins, chemicals that play a vital role in research to understand the development and functioning of the nervous system. Cyanobacteria can also be a source of novel ligases, polymerases, and restriction endonucleases with unusual substrate specificities and unique catalytic activities [23].

CYANOBACTERIAL GENETICS

In view of the immense biotechnological potentials of cyanobacteria, it is obvious that the studies on genetics and genetic engineering are important. The identification and characterization of the classic mechanisms for genetic recombination in cyanobacteria have lagged behind in comparison to other prokaryotes. This in part is due to the little information available on the general physiology of these organisms, the difficulties in culturing many isolates, the impossibility of growing single colonies and the absence of well-defined stable mutants. Indeed, for a long time cyanobacteria have been thought to be devoid of any gene transfer system, until Kumar (1962) [24] reported the isolation of double-resistant colonies by mixing cultures of Synechococcus mutants resistant to either streptomycin or penicillin.

The first clear evidence in favour of the occurrence of DNA-mediated transformation in unicellular cyanobacteria was reported for Synechococcus 7943 [25]. It is now clear that most of the powerful techniques of bacterial

genetics are applicable to cyanobacteria. The efficient systems of gene transfer have opened the door for gene-cloning in cyanobacteria and for the construction of strains with favourable characteristics. Methods are available to increase and control the expression of genes of interest in cyanobacteria. A wide variety of genes have been cloned and characterized, particularly in nitrogen fixing cyanobacteria. These organisms are of special interest as recipients for plant genes and possible introduction of useful genetic information into plants. There are a few reviews on genetic techniques as applied to cyanobacteria [26-30].

Plasmids

Plasmids have been reported in most of the unicellular [31-36] and many of the filamentous [37-44] cyanobacteria. All plasmids so far characterized appear to be genetically cryptic and their role in physiology, ecology and evolution of cyanobacteria is still not known [33, 35, 37, 45]. The absence of plasmids in some cyanobacteria [46] and a low copy number in a few are reported. Plasmids can serve as vehicles to shuttle the genetic material amongst cyanobacteria [47-49].

DNA Transfer

Foreign DNA can be introduced into cyanobacteria by various means [26, 50-52]. Mutations can be made tagged with transposons [53, 54], which are DNA sequences that can insert themselves at one of the multiple positions in foreign DNA, or mutations directed to specific sites within genes *[55,* 56]. Identification of important genes by complementation *[57]* of mutations induced by classical means [27, *55]* is possible. Fusion of the regulatory region of one gene with an open reading frame of another, to gain control over the latter's expression [58, 59] has been carried out. The genome of one strain has been extensively mapped [60].

Mutagenesis

* Tandeau de Marsac et al (1982) [61] identified a gene involved in methionine biosynthesis by transposition of transposon Tn901 (encoding ampicillin resistance) into the chromosome of Synechococcus PCC 7942. Several transposons have been constructed for special purposes, for e.g. placing lux AB at random in the chromosome [54].

* Some methods of mutagenesis involve integration of foreign DNA into the chromosome called recombinational mutagenesis [62-64].

* Ectopic mutagenesis was used by Buzby et al (1985) [63] to produce mutations at random sites in the chromosome of Synechococcus PCC 7002. This method was used to show that all three of the psbA genes in Synechococcus PCC 7942 are functional [62].

* Site-directed mutagenesis of genes cloned in E.Coli by repair of a single strand hybridized to a mismatched oligomer, has been described [65]. Transformation with genomic DNA coding for specific genes of the photosystem II reaction centre, interrupted by heterologous insertions or modified by site-directed mutagenesis, has been performed in Synechocystis 6803 [66, 67].

Conjugation and Electroporation

DNA from E.coli is introduced into cyanobacteria [68], using the broad host range conjugal apparatus of an IncP plasmid, such as RP4 [69], originally isolated from Pseudomonas. The possibility of DNA transfer from E.Coli to cyanobacteria, via conjugation, was then tested in a number of strains of Nostoc and in Fischerella UTEX 1829 [70]. Hybrid plasmid carrying the genes for bacterial luciferase lux A and lux B with the promoter for structural genes for ribulose biphosphate carboxylase or nitrogenase inserted upstream from luciferase genes through conjugation and monitoring the production of light in exconjugants has confirmed expression of bacterial luciferase in Anabaena [71].

Electroporation [52] is a simple alternative to conjugation. This technique permits total control over the form of DNA introduced into the target cyanobacterium. Interspecific conjugation requires the entry of DNA from E.Coli to cyanobacterium in the form of a plasmid or linear DNA [72], whereas electroporation permits transfer of DNA taken directly from a cyanobacterial source, DNA isolated from E.Coli or manipulated in_ vitro.

Reporter genes

Recent developments in gene cloning techniques in cyanobacteria permit the use of the cat [58], lacZ [63] and lux [71] genes, previously applied to other cell systems [73, 74]. Their respective gene products, chloramphenicol acetyltransferase, beta-galactosidase and luciferase can be assayed in these organisms [58, 63, 71].

DNA hybridization

The molecular genetics of the organization of regulation of nif (nitrogen fixation) genes in cyanobacteria initiated in Anabaena species PCC 7120 used DNA hybridization techniques with Klebsiella pneumoniae nif probes and DNA from vegetative cells *[75]*. Four genes involved in nitrogen fixation in Anabaena PCC 7120 have been identified [75]: nif H, nif D and nif K encoding the polypeptide components of the nitrogenase complex and nif 5, a gene involved in Klebsiella pneumoniae in the maturation of nitrogenase. Using Anabaena PCC 7120 nif genes as hybridization probes, the nif structural gene arrangement has been studied in several other cyanobacteria including unicellular and filamentous strains [76-81].

Cloning and expression of cyanobacterial genes

Many cyanobacterial genes so far cloned **(Table** 4), were sequenced and expressed. Different strategies were employed to identify such genes and their products.

Table 4 : Cloned cyanobacterial genes

Table 4 : Cloned cyanobacterial genes

Gene	Coding for	Organism
Genes for photosynthesis and CO_2 fixation		
rbcL.	Large subunit of ribulose-bisphosphate caroboxylase	Anabaena 7120
		Chlorogloeopsis 6912
		Spirulina
		Synechococcus 6301
rbcS	Small subunit of ribulose-bisphosphate carboxylase	Anabaena 7120
		Chlorogloeopsis 6912
		Spirulina
		Synechococcus 6301
psaA,B	P700 chlorophyll a apoproteins	Synechococcus 7002
psbA	32kd (or Q_B) protein	Anabaena 7120
		Calothrix 7601
		Synechococcus 7942
psbB	CP-47 protein	Synechocystis 6803
woxA	Extrinsic 33kd protein	Synechococcus 7942
petF	Ferredoxin I	Anabaena
		Anabaena 7120
		Synechococcus 7942
ppc	Phosphoenolpyruvate carboxylase	Synechococcus 6301
trxA	Thioredoxin	Anabaena 7119
atp	Proteins of the ATP-synthase complex	Synechococcus 6301
atpB	Beta-subunit of ATPase	Anabaena 7120
atpE	Epsilon-subunit of ATPase	Anabaena 7120
cpcA	Alpha-subunit of C-phycocyanin	Anabaena 7120
		Synechococcus 7002
		Calothrix 7601
		Synechococcus 7942
cpcB	Beta-subunit of C-phycocyanin	Anabaena 7120
		Synechococcus 7002
		Calothrix 7601
		Synechococcus 6301
		Synechococcus 7942
cpeA	Alpha-subunit of phycoerythrin	Calothrix 7601
cpeB	Beta-subunit of phycoerythrin	Calothrix 7601
apcA	Alpha-subunit of allophycocyanin	Synechococcus 6301
apcB	Beta-subunit of allophycocyanin	Synechococcus 6301
cpcC D,E	Linker polypeptides of phycobili-somes	Anabaena 7120
lpcA B,C	Linker polypeptides of phycobili-somes	Calothrix 7601
apcC	Linker polypeptides of phycobili-somes	Synechococcus 6301

Genes for nitrogen fixation or metabolism

nifD	Alpha-subunit of dinitrogenase	Anabaena 7120
nifK	Beta-subunit of dinitrogenase	Anabaena 7120
nifH	Nitrogenase reductase	Anabaena 7120
xisA	Excision enzyme	Anabaena 7120
glnA	Glutamine synthetase	Anabaena 7120
		Spirulina
nar	Nitrate reductase	Synechococcus 7942

Genes for the protein synthetic apparatus

rrnA,B	23S rRNA	Synechococcus 6301
	16S rRNA	Synechococcus 6301
	5S rRNA	Synechococcus 6715
		Synechococcus 6301
trnI	$tRNA_{Ile}$	Synechococcus 6301
trnA	$tRNA_{Ala}$	Synechococcus 6301
tufA	Elongation factor Tu	Spirulina
fus	Elongation factor G	Spirulina
tsf	Elongation factor Ts	Spirulina
rps12	Ribosomal protein S12	Spirulina
rps7	Ribosomal protein S7	Spirulina
rps2	Ribosomal protein S2	Spirulina

Other genes

gvpA B,C	Gas vesicle proteins	Calothrix 7601
recA	rec A protein	Anabaena
		Synechococcus 7002
		Synechocystis 6308
arg		Synechococcus 7002
leu		Synechococcus 7002
met		Synechococcus 7942
thi1		Synechococcus 7942

Ref.: Houmard, J. and Tandeau de Marsac, N. (1988). Methods in Enzymology 167: 808-847.

the same organism, such as the ribosomal RNAs that were utilized to isolate the corresponding genes in Synechococcus 6301 [82]. Probes from the same gene cloned from another cyanobacterium can also be used, for example, the gene for glutamine synthetase of Spirulina was isolated employing a probe derived from the cloned Anabaena gene [83]. However, so far, such probes are rarely available and probes from bacteria have been successfully utilized to identify the corresponding genes on cyanobacterial DNA, making use of the similarity in the nucleotide sequence of bacterial and cyanobacterial genes. This approach was used for isolating the glutamine synthetase gene of Anabaena [84], a recA gene of Synechococcus 7002 [85], the genes for the elongation factors [86] and three ribosomal proteins of Spirulina [87] and that for thioredoxin of Anabaena [88]. Similarly, the cloned nif genes from Klebsiella pneumoniae have been extensively utilized to identify some of the genes involved in nitrogen fixation in many cyanobacteria, e.g. Anabaena [75, 80, 89].

It is possible to identify genes in cyanobacteria by using probes from the chloroplasts taking advantage of the similarities existing in the genetic organization of chloroplasts and cyanobacteria. Thus, the genes for the apoproteins of the phycobiliproteins cloned from the chloroplast DNA of Cyanophora paradoxa allowed the identification of the genes for the phycocyanins in Calothrix [90] and the allophycocyanins in Synechococcus 6301 [91]. Similarly, the gene for the large subunit of ribulose-biphosphate carboxylase from spinach or Chlamydomonas reinhardtii chloroplasts was used for isolating the corresponding gene in Synechococcus 6301 [92] and in Spirulina [93] and that for the 32 kd protein from spinach and maize for Anabaena [94], Calothrix 7601, Nostoc and Synechococcus 7942 [95]. In the case of the gene for the small subunit of the carboxylase that is coded in the nuclear DNA in eukaryotes, probes from the nuclear genes from pea and soybean allowed the identification of this gene in Synechococcus 6301 [96] and Spirulina [93] respectively.

Oligonucleotides, corresponding to a part of the gene, are synthesized and utilized as probes to identify the DNA fragment carrying the gene for such

a protein. This approach was followed for the phycoerythrin genes in Calothrix 7601 [97], for phycocyanins in Synechococcus 7942 [98] and Synechococcus 7002 [99] and for the linker polypeptide in Calothrix [100]. Similarly, synthetic probes allowed the identification of the genes for the gas vesicle protein in Calothrix [101], and that for ferredoxin I in Anabaena [102] and Synechococcus 7942 [103].

Complementing well-defined mutations in bacteria by a ' shotgun' type of experiment is another approach. The glutamine synthetase gene of Anabaena [84] and Spirulina [83], the recA gene of Synechocystis 6308 [104] and some genes of the leucine, threonine and proline pathways in Synechococcus 7002 [105] were identified using this method.

Establishment of expression of cloned cyanobacterial genes by detection of an enzymatic activity, absent in the bacterial cells, but present after transformation with plasmids carrying fragments of cyanobacterial DNA, has been achieved in the case of ribulose-biphosphate carboxylase genes [106-110]. The expression of the cyanobacterial genes in ~ 'maxicells' [84, 85, 111] or 'minicells' [87, 93] followed by confirmation of the authenticity of the protein either by immunology [84, 111] or by comparison of the patterns obtained on partial proteolytic digestion of the protein isolated from the transformed bacterial cells and that isolated from the cyanobacteria [93] are the other approaches that have been shown. In some cases, plasmids carrying cyanobacterial genes were transcribed and translated in in vitro systems resulting in the production of proteins with the same electrophoretic mobility of the cyanobacterial proteins [83, 112].

THE SPIRULINA

The genus Spirulina of the Oscillatoriaceae family consists of filamentous cyanobacteria characterized by spiral-shaped chains of cells (trichomes) enclosed in a thin sheath. The cell dimension, degree of coiling, and length of the filaments (-300 - 500 μ long and 8 *p* wide) vary with the species. The latter characteristics may also vary within a species depending upon growth and environmental conditions [113].

The unbranched, gliding trichomes elongate by intercalary cell division. Reproduction is accomplished by breakage of the trichome through the "sacrifice" of an intercalary cell that loses its cytoplasm and is converted into a necridium. Trichomes break at the necridia, giving rise to short chains of cells. These daughter chains, sometimes called hormogonia, are distinguishable from the mature trichomes by their lack of motility, smaller cell size, and different cell morphology [113].

Spirulina does not fix atmospheric nitrogen. Except for S.labyrinthyformis, (a thermophilic species, isolated from hot springs), which is capable of facultative anoxygenic photosynthesis at the expense of sulphides [114], all other members of the genus Spirulina are obligate photoautotrophs endowed with oxygenic photosynthesis.

ORIGIN

After the isolation in 1827 of the first species, S.oscillariodes, from a freshwater stream [115], several species of Spirulina have been reported from quite different environments: brackish waters, sea, tidal pools, and saline ponds. Members of this genus have also been found in subarctic waters, tropical lagoons, and hot springs. Thus, these organisms appear to be ubiquitous, being capable of adaptation to extreme environmental conditions. The most illuminating example of this adaptability is perhaps that of the alkalophilic species that grow abundantly in waters whose alkalinity is as high as pH 11, where most of the microorganisms do not thrive. Detailed investigations of the phytoplankton of the small lakes or temporary ponds near Lake Chad [116-118] or the lakes in the Rift Valley in East Africa [119] have shown that Spirulina is frequently the major organism and, sometimes, the only one in the microflora [120].

HISTORY

Even more interesting is the well-documented utilization of Spirulina as a source of food for humans. Two independent reports from France in 1940 [121] and Belgium in 1967 [122], indicated that dried S.platensis was consumed by the native population of the sub-Saharan region of Kanem, northeast of Lake Chad. Indeed, the dried mats of S.platensis in the shape of biscuits or flakes were sold as "dihe" in the markets of the capital city of Fort Lamy. "Dihe" was used to prepare the sauces for the millet meal eaten by the Kanenbou population. The spiral shape of the trichomes and the presence of abundant gas vacuoles resulted in the formation of large floating mats of S.platensis that the wind brought to the shores of the small lakes or ponds around Lake Chad. The mats were collected, laid on the shores and sun-dried. The dried paste, broken in rectangular biscuits, represented "dihe" that, in 1976, was still consumed extensively [123].

The potentiality of Spirulina as a food for humans was exploited possibly in ancient Mexico as well. Historical records reveal that (at the time of conquest of Mexico by Spain), biscuits analogous to "dihe" were sold in the Mexican markets [124]. The biscuits were prepared, as in the case of "dihe", by sun-drying mats of S.maxima, that grows abundantly in the highly alkaline waters of Lake Texcoco near what is now Mexico City. Currently, the first large-scale factory for the production of Spirulina operates on this lake. Thus, it appears that two populations, at considerable distance in time and space, discovered independently the nutritional quality of Spirulina. Their discoveries are indicative of a significant nutritional value of the organism.

COMPOSITION AND NUTRITIONAL PROPERTIES

Various studies on the chemical composition of the biomass of S.platensis and S.maxima, document an unusually high protein content. Such a high level of protein in Spirulina is uncommon even in the microbial world, being surpassed only by certain bacteria like Cellulomonas, which is recorded

to have protein levels above 80% of the dry weight [125, 126]. However, this high protein content in those bacteria is accompanied by high nucleic acid content, which is medically undesirable. Excess nucleic acids [127, 128] result in uric acid accumulation due to purine catabolism, which may in turn lead to pathological conditions such as gout. In Spirulina, the concentration of nucleic acid is always below 5% of the dry weight and is thus advantageous.

Research conducted on Spirulina by the World Health Organisation and scientists in the United States, France, West Germany, Mexico, Vietnam and Japan confirmed that it has a blend of nutrients that no single plant source can provide. The **protein** content of Spirulina is higher than that of any other food [129, 130] **(Table 5a).** The NPU value of Spirulina is comparable to that of other vegetarian sources indicating good aminoacid composition, digestibility and biological value. The **amino acid** profile of Spirulina protein is impressive. Indeed, with the exception of cysteine and lysine, which are somewhat lower than that of the standard protein (FAO), all the other essential amino acids are present in adequate concentrations [131-135]. The potential use of Spirulina as a source of protein for human consumption has been widely recognized [136].

The **carbohydrates** of Spirulina are easily digested owing to the absence of cellulose. Further, the absence of free sugars, makes it an ideal food supplement for diabetes, obesity and such other conditions. Spirulina has yet another merit in its **lipid** composition, being cholesterol free and rich in **poly-unsaturated essential fatty acids** [137], which is suitable for conditions like aetherosclerosis, obesity and blood pressure. Additionally, this alga is rich in **gamma linolenic acid,** a precursor for prostaglandin biosynthesis and thus has medical implications [130]. In short, the composition of Spirulina makes it a low-calorie, low-fat, cholesterol-free source of protein.

Spirulina has an excellent blend of **vitamins,** including Vitamins A, B_l, B_2, B_6, B_{12}, E and H (biotin). Spirulina is the richest vegetarian source of Vit_o B_{12} available (1 gram of powder supplies 53% of the RDA) [130]. It contains 21% of the RDA in the case of thiamin and riboflavin [130]. Spirulina

Table 5a: Protein content of Spirulina as compared to other food

Dehydrated Spirulina powder	68%
Whole dried egg	47%
Dry soyabean powder	37%
Powdered milk	36%
Dried peanuts	27%
Chicken	22%
Fish	19%
Whole fresh egg	15%
Fresh cow's milk	4%

has a ß-carotene (pro-vitamin A) content (0.1% of the dry weight), which is 20 times higher than that of carrots. Its folic acid and Vit_o B12 contents make it a good therapeutic food for anemia. The **mineral** make-up of Spirulina is attractive too. It has 12 times higher levels of iron than that of any other food and is also rich in magnesium, potassium and other trace elements [130]. Spirulina being particularly rich in iron and calcium, is good for blood rejuvenation and healthy functioning of bones and teeth.

The nutritional value of Spirulina has been well documented by detailed studies carried out by UNIDO; Central Food Technological Research Institute, Mysore; Shri A.M.M.Murugappa Chettiar Research Centre Madras, and several international agencies. The composition of spray-dried Spirulina is given in **Table 5b.**

IN SELF-CARE STRATEGIES

Spirulina has been called a ' super food' because of its concentrated nutrients. It is a compact well-balanced source of nutrition unsurpassed by any other food known to mankind. Spirulina is most effective when used in a natural diet as a part of the self-care strategy.

Spirulina can help dieters eat lighter as part of a natural weight control plan. As a tonic, it is a good adjuvant for, growing children and adolescents. It is ideal for fasting and for body cleansing programmes. Athletes, body builders and outdoor activists use Spirulina as a foundation food for stamina and peak performance. Older people on restricted diets find it easy to digest. Vegetarians appreciate its concentrated vegetable proteins and vitamins. Today, Spirulina is a part of US Olympic team's food for energy and is popular among busy executives in Japan for stress relieving. It is being taken as a slimming diet in Germany and as a perfect whole-food supplement for pregnant women and nursing mothers in Vietnam and India. It is an excellent food in diet therapy for eyes, skin, anaemic and hypercholesterolemic conditions. It is used in cosmetics as a source of natural colorant and for skin care.

Table Sb : Composition of spray-dried Sp irulina (Constituents per 100 grams)

Major constituents	
Calories	346
Protein	68%
Fat	7%
Crude fibre	9%
Carbohydrates	16%
Vitamins	
Beta-carotene	320,000 I.U.
Biotin	0.22 mg
Cyanocobalamin (B12)	65.7 ug
Folic acid	17.6 ug
Other B-complex vitamins	9.2 ug
Tocopherol (E)	0.73 I.U.
Minerals	
Calcium	658 mg
Phosphorous	977 mg
Iron	48 mg
Sodium	796 mg
Potassium	1,140 mg
Essential amino acids	
Lysine	2.99%
Cystine	0.47%
Methionine	1.38%
Phenylalanine	2.87%
Threonine	3.04%

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INDIAN STANDARD : FOOD GRADE SPECIFICATION

This alga has been approved by FDA of the U.S. for being used as a natural food. Spirulina of species platensis, maxima and fusiformis are at present used for the commercial production of the food grade alga. Though there are variations in the details of the manufacturing processes, the production of food grade alga basically involves the following steps.

* Selection of suitable strains of alga;

* Preparation of nutrient medium and specified culture conditions;

* Cultivation in open culture ponds under defined conditions;

* Harvesting alga by filtration and washing to get rid of residual nutrients;

* Suitable drying to flakes or fine powder.

The Indian Standard (Bureau of Indian Standards, August 1990) has specified compositional, mineral and vitamin requirements for food grade Spirulina.

PRODUCTION PLANTS

The large production sites of Spirulina biomass are currently located in the American Southwest, Hawaii, Mexico, and the Far East (mainly Thailand)., Although some low grade products are produced in an extensive mode, i.e., using unlined deep ponds, with no mixing or other methods to induce turbulent flow, the main production of high grade product is in raceway type ponds

In the past twenty years, considerable progress has been made in maintaining a continuous production of Spirulina biomass in outdoor cultures. The mode of production is based on the principles of a turbidostat culture,

where a constant cell concentration is maintained in the ponds, usually in the range of *400-600* mg/L of dry weight, by daily harvesting. The clarified medium, enriched with nutrients (along with short Spirulina filaments) is returned to the pond. The harvested biomass, which is a slurry of 8-10%, is further subjected to vacuum filtration and then to the last step of drying. In **Table 6,** a summary of the current production sites, along with their actual productivity is presented. The world annual production of Spirulina is about 1000 tons.

In India, production of Spirulina was started ten years ago by Shri A.M.M. Murugappa Chettiar Research Centre, Madras, which set up a semi-commercial unit at Saveriarpuram in Tamil Nadu with a production capacity of about 7 tons/year. Many commercial units have come up in various parts of the country in the past few years. In spite of the availability of complete know-how and favourable climatic conditions, the production and utilization of Spirulina in India are yet to make a significant industrial impact.

The Central Food Technological Research Institute, Mysore has developed a technology for large scale cultivation (Fig. 2A) and down-stream processing of Spirulina and has been instrumental in popularizing its commercial applications in India. Several formulations of Spirulina for use in food, feed, biocosmetics and medicine have been developed in India (Fig. 2B).

PRODUCTION COSTS

In the early 1980's, Sosa Texcoco Ltd. (Mexico) was selling around *500* tons of Spirulina annually at a wholesome price of about $ to/kg to the European market. Due to vigorous promotional campaigns, marketing strategies and advertisements claiming Spirulina as an effective appetite suppressant, the price reached $ 140 to 160/kg in 1983. In India the price of food grade spray dried Spirulina powder is Rs. 1000/kg and freeze dried powder is Rs. 5000 - 6000/kg.

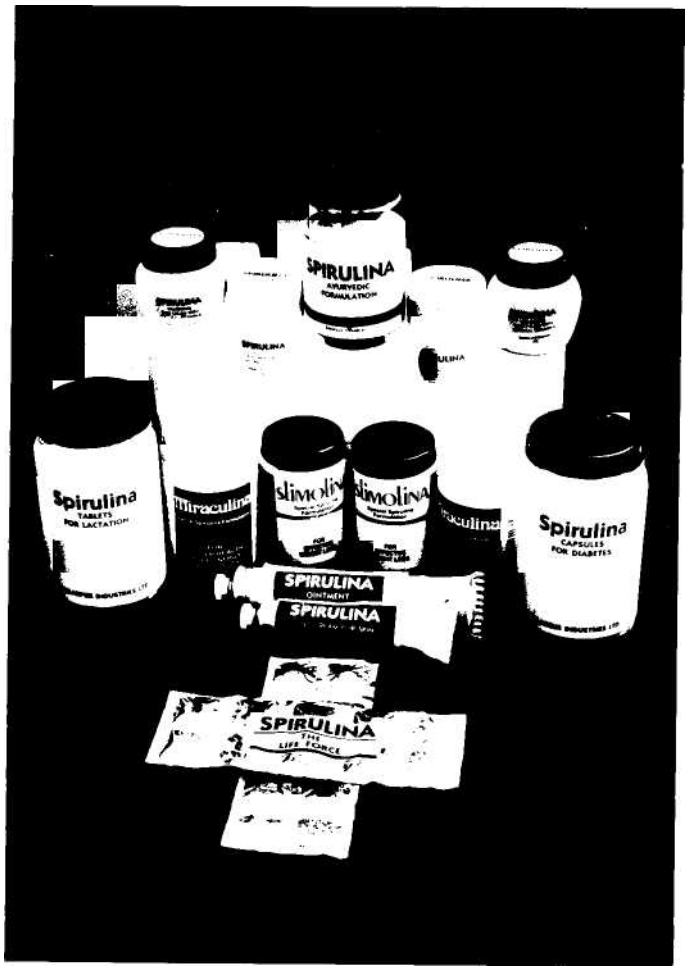
Table 6 : Spirulina production plants

Company	Location	surface m x10	Species utilized	Yearly production (tons dry wt)
Sosa Texcoco	Lake Texcoco, Mexico	120	S.maxima	330
		50	S.platensis	130
Proteus	Calipatria, Cal., U.S.	20	S.platensis	50-60
Spirutec	Chandler, Ariz., U.S.	13	S.platensis	90
Earth Rise Farms	California, U.S.			
Cyanotech	Hawaii, U.S.	27	S.platensis	80-90
Siam Algae	Bangkok, Thailand	18	S.siamensis	60-100
		30	S.platensis	50-60
Blue Continent	Taiwan (North)			
Chlorella Spirulina Research	Taiwan (North)	66	S.platensis	120-150
Laboratories Tung Hai Chlorella	Taiwan (Central)	30	S.platensis	50-60
Industries Far East Microalgae	Taiwan (South)	33	S.platensis	50-60
Industries Nippon Spirulina	Miyako Isl., Japan	13.5	S.platensis	10-40
	Elat, Israel	12	S.platensis	10
Hills-Koor Algae Production	Ein-Yahav, Israel	5.5	S.platensis	20
Microal~ae. ProductIon New Ambadi Estates		5	S.fusiformis	10
	India			
Ballarpur Industries	India	_	S.platensis	250

All data except for India from
Richmond, A. 1988. Spirulina. In: M.A. Borowitzka and L.J. Borowitzka (eds), Microalgal Biotechnology, pp. 85-121. Cambridge University Press, Cambridge.

Data for India from
Seshadri, C.V. 1992. Large scale production systems. In: C.V. Seshadri and N. Jeeji Bai (eds), Spirulina ETTA Nat. Symp. MCRC, Madras, pp. 22-26.

B. Formulations of Spirulina developed in India



The cost of production is estimated to range from US $ 15-30/kg dry weight. The lower figure is based on a "feed" grade product. The sun-dried product contains a high ash and low chlorophyll content. The demand for such a product is continually growing, as its relatively low price makes it more attractive to the feed industry as an additive to fish and chicken feed. A starch producing factory in Thailand has started to use its water refuse from an anaerobic digester to grow Spirulina and then sun-dry the product. This is a very important step towards the production of Spirulina, not only as a health food product, but for a much larger market for feed additives [138].

In India, with the long term yields of 8-10 g/m /d and high nutrient inputs and drying costs, food grade Spirulina of good quality can be produced for Rs. 350-500/kg.

MARKET AND APPLICAnON

The biomass produced is mainly sold to the health food market in the form of powder or tablets. Attempts have been made by Proteus (a marketing company mainly associated with Earthrise Farms in the U.S.) to incorporate Spirulina into a variety of food products such as granola bars and various kinds of pasta. In Mexico and China (subsidized by the government), Spirulina powder is added to children's foods such as biscuits, chocolates, etc. [139].

Another available product is a protein extracted from Spirulina, containing mainly the blue pigment phycocyanin and marketed under the "Lina Blue" brand name [140, 141]. The product is mainly used as a colorant for the food market, as an edible dye for ice creams and as a natural dye in the cosmetic industry. The main problem is that the pigment is light sensitive and special care has to be taken in handling the dye to protect it from bleaching. A full account of the applications of Spirulina in human nutrition and various therapeutic effects was given [130]. In **Table 7,** some of these applications are summarized. It should be pointed out that none of these applications have been permitted by the U.S.-FDA as a proven "claim" for. marketing and more experimental work has to be performed before such approval is given.

Table 7 : Clinical applications of Spirulina Summary of scientific research

	Subject	Country, Year
Lowering cholesterol	humans	Japan, **1986**
Lowering cholesterol	rats	India, **1983**
Lowering cholesterol	rats	Japan, **1984**
Reducing oral cancer	hamsters	USA, **1986**
Building healthy lactobacillus	rats	Japan, 1987
Reducing kidney poisons from drugs and heavy metals	Japan,	1988
Treating infirmities with GLA	humans	Spain, 1987
Anti-liver cancer tumor and stimulating immune system	mice	Japan, 1982
	rats	USA, 1986
High iron bioavailability	rats	Japan, 1982
Correcting iron anemia	humans	Japan, 1978
Correcting iron anemia	humans	Mexico, 1973
Recovering from malnutrition	humans	Togo, 1986
Recovering from malnutrition	humans	Romonia, , 1984
Treating nutritional deficiencies	humans	China, 1987
Treating nutritional deficiencies	humans	Germany, , 1986
Weight lowering effect	humans	France, 1967
Treat~ng external wounds	humans	Japan, 1977
TreatIng external wounds	microbial cells	Puerto Rico, 1970
Infection (antibiotic action)	microbial cells	India 1978
Infection (antibiotic action)		

Data from
Henrickson, R. 1989. Earth Food Spirulina, pp. 180. Ronore Enterprises Inc., Laguna Beach, California.

A COMPARISON WITH OTHER MICROALGAE

At least two other microalgae are now sold: Chlorella (a green alga), as a health food supplement and Dunaliella (a red alga) for p-carotene.

Chlorella is a unicellular green microalga that was the first to be used in applied research. In the late 1960's, the Taiwan Chlorella Company started the large scale production of Chlorella biomass, and ten years later, at least thirty different companies were involved in production and marketing of various Chlorella-based products. Its total annual production in the late 1970's was estimated to be about 1000 tons, having a potential of at least double that amount [142, 143]. The market for Chlorella products is limited to the Far East, mainly Japan. Three drawbacks limit its potential as a new food source for the developing world.

1) Chlorella culture is easily contaminated by undesirable weed algae, unlike Spirulina which flourishes in highly alkaline water unfriendly to other algae. Chlorella must be cultivated in individual culture batches which is not cost effective.

2) Unlike Spirulina, Chlorella cells cannot be harvested by a simple cloth or screen. Expensive centrifuges are required to separate the cells from pond water which is unsuitable for small scale systems in villages and local areas.

3) Chlorella, unlike Spirulina, has a hard cellulosic cell wall which is not easily assimilable. The cell breaking procedures would be difficult to adopt in a low technology system [130].

Dunaliella, a unicellular bi-flagellated green alga known for its unique osmoregulation mechanism are isolated from salt lakes [144, 145]. It was first suggested as a commercial source for glycerol since it accumulates glycerol (upto 50% of the cell mass) as an osmoregulant [144]. Further, Dunaliella is known for very high accumulation of ß-carotene (8-12% on a dry

weight basis} under stress conditions [146]. But, this alga is not ideal for human consumption due to its high glycerol content. These comparisons again focus the superiority of Spirulina.

IN BASIC RESEARCH

Although cyanobacteria are the most primitive of the oxygen-producing photosynthetic organisms and are separated from higher plants by an exceedingly big evolutionary distance, they possess a photosynthetic apparatus that is remarkably similar to that present in higher plant chloroplasts *[147]*. However, because cyanobacteria are prokaryotes, their photosynthetic apparatus is not organized in a specific organelle, the chloroplast, but is dispersed throughout the cell. Furthermore, as in the case of red algae, phycobiliproteins replace the chlorophyll-b-protein complex that acts as a light-harvesting antenna of photosystem II in other algae and higher plants.

The phycocyanins, biliproteins involved in the light-harvesting reactions, have been resolved by gel-electrophoresis in S.platensis and S.maxima [148] and isolated from the former [149]. Both c-phycocyanin and allophycocyanin appear to be oligomeric complexes composed of atleast two different subunits.

Ribulose-1,5-biphosphate carboxylase, accounting for ca. 12% of the soluble protein of S.platensis and S.maxima, was purified and partially characterized [150]. By gel electrophoresis the molecular weight of the enzyme was estimated to be ca. 500,000, a value similar to that reported for the enzyme isolated from chloroplasts of higher plants, unicellular algae and some cyanobacteria [151].

Cytochrome-c55$_4$' a cytochrome with high redox potential that links photosystems I and II, has been purified from S.platensis [152] and S.maxima [153]. The molecular weight of the protein was found to be ca. 10,000, like that of cytochrome-c554 from other cyanobacteria, unicellular algae, and higher plants. Another cytochrome involved in photosynthesis, cytochrome-f,

was purified from S.maxima [153-155] and S.platensis [156], with a molecular weight of ca. 38,000, a value close to that of cytochrome-f isolated from spinach chloroplasts.

Ferredoxin, one of the electron carriers of photosynthesis, was purified from S.maxima [157] and sequenced from S.maxima [158] and S.platensis [159]. As the protein isolated from higher plants, S.maxima ferredoxin contained two atoms of Fe and two atoms of sulfur per mole. Optical adsorption, oxygen evolution and other spectral characteristics confirmed the close similarity existing between the ferredoxin isolated from the cyanobacterium and that isolated from higher plants and algal chloroplasts. Ferredoxin II, another ferredoxin characterized by a different redox potential and present in smaller amounts, was isolated in a subsequent investigation [153, 160].

A preliminary report of a cyanide-insensitive superoxide dismutase from S.platensis indicated the presence of Fe, as in some bacterial and cyanobacterial dismutases in contrast to Cu or Zn found in SOD from chloroplasts [161, 162].

The only data on the transport of inorganic nutrients in Spirulina is regarding sulfur. S.platensis appears to possess an active, energy-dependent transport system for sulfate. Under photo-autotrophic conditions, probably two sulfate permeases are present. One permease is constitutive, as found for the only other cyanobacterium studied, Anacystis nidulans, whereas the other is inducible like in heterotrophic bacteria, fungi, and some higher plants [163].

A number 01 mutants of S.platensis resistant to two analogs of phenylalanine, methionine, proline and tryptophan were isolated and partially characterized [164]. A few mutants appear to be resistant to one analog only, whereas the majority seem to be resistant simultaneously to the analogs of phenylalanine, methionine, and proline but not of tryptophan. All the cross-resistant mutants analyzed appear to overproduce the respective parental amino acids and thus became resistant to the analogs by reducing their uptake into the cells and their incorporation into protein [165].

GENETICS

There is no information on the mechanisms for genetic recombination in Spirulina or in any of the Oscillatoriaceae. Spheroplast preparation from S.platensis [166] has been reported. If methods are standardized for isolating good regenerable protoplasts, it will be of considerable help in attempting heterologous fusions, a technique successfully used for other prokaryotes, like streptomycetes, which are recalcitrant to conventional genetic recombination. Numerous attempts have been made to isolate protoplasts from cyanobacteria due to their effectiveness in direct DNA uptake or in fusion studies for desirable genetic combinations. However, there have been no reports available on the isolation of viable protoplasts.

Spirulina has simpler growth requirements than bacteria, is non-pathogenic and therefore, is ideal for genetic studies. So far no phage infecting Spirulina or other Oscillatoriaceae has been isolated [167, 168], nor have plasmids been found in S.maxima and S.platensis. However, plasmids with selectable markers constructed for other cyanobacteria (e.g. S.nidulans [169]) which can transform other bacteria may be utilized for Spirulina.

The genomic sizes of 128 strains representing all major taxonomic groups of cyanobacteria have been determined by renaturation kinetics [170]. The genomic sizes could be grouped into four classes that could correspond to a progressive duplicaton of an "ancestral genome" of ca. 1.2 x 109 daltons to give genomes two, three, four, and six times this ancestral genome. The DNA base compositions of the two isolates of Spirulina analyzed have been reported to be 44 and 52 mol% guanosine plus cytosine (G-C) [171].

The genetics of Spirulina is still in its infancy. But with the popularity that this alga has been gaining, it is expected that the genetic engineering of Spirulina would get a new dimension in order to develop improved strains for commerical exploitation.

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OBJECTIVES

It is very obvious that the nutritional and economic potentials of Spirulina are immense. This study is aimed at a better understanding of this organism with the following objectives in view, for its exploitation as a source of food/feed and valuable commercial products.

* To develop a germplasm bank of various Spirulina strains followed by thorough screening of the strains for identifying suitable strains for commercial exploitation

* To obtain a better knowledge of the nature of a few important phyto-chemicals and proteins of high value in Spirulina followed by attempts to enhance their levels in the cells by various physical and chemical methods of induction

* To develop protocols for obtaining regenerable protoplasts in good yield for direct transformation

* To make preliminary attempts on direct transformation of Spirulina using shuttle plasmid vectors.

The work conducted is presented in the following chapters.

1. Collection, maintenance and screening of Spirulina strains
2. Carotenoids of Spirulina
3. Biliproteins from Spirulina
4. Superoxide dismutase of Spirulina
5. Attempts on genetic improvement of Spirulina for value addition

CHAPTER 1

COLLECTION, MAINTENANCE AND SCREENING OF *SPIRULINA* STRAINS

SUMMARY

A germplasm was developed where 15 different strains of Spirulina collected from various parts of India as well as from other countries were purified and maintained as axenic cultures. The cultures were grown in Zarrouk's medium either on agar slants or in liquid broth. They were subcultured periodically. Imipenem, a broad-spectrum J3-lactam antibiotic which inhibits peptidoglycan biosynthesis, was found to be superior to a few other J3-lactam antibiotics in purifying Spirulina cultures. This reduced the number of heterotrophic bacterial contaminants associated with freshly isolated Spirulina to a level which facilitated the production of axenic Spirulina cultures.

A screening of the strains was carried out to select the elite strains in terms of biomass, protein yield, pigment levels <p-carotene, phycocyanin) and superoxide dismutase (SOD) activity. FTCC 06 strain was found to be the best for biomass, protein and phycocyanin production. FTCC 03 had the highest SOD activity. J3-carotene accumulation was more in FTCC 13 as compared to other strains.

Despite the existence of morphologically diverse cyanobacteria in a wide variety of terrestrial and aquatic habitats, research on these bacteria is restricted to a relatively few representatives due to the difficulties encountered in both isolation and subsequent purification of these organisms. The techniques normally used to isolate cyanobacteria severely limit the number of cyanobacterial species which can be readily cultured [1].

Due to the pioneering work of Gerloff et al [2], M.B. Allen [3], Kratz and Myers [4], Hughes et al [5], van Baalen [6] and M.M. Allen [7] in the early 1950s to late 1960s, the isolation and identification of cyanobacteria was made possible. Later studies resulted in a good number of axenic cyanobacteria in various major and minor culture collections spread over different countries. The isolates originated from a wide range of habitats. Methods for the isolation and purification of cyanobacteria have been reviewed by Castenholz [8] for thermophiles, by Walsby [9] for planktonic species, by Waterbury and Stanier [10] for marine and hypersaline organisms, and by Rippka *et_* al. [11] for other organisms of diverse origin.

The tedious and time-consuming work of producing axenic cyanobacterial cultures often gives low rate of success. Various approaches have been taken to develop more-efficient methods to purify contaminated cyanobacteria. These include mechanical separations of the cyanobacteria and bacterial contaminants by micromanipulation [12], differential filtration [13, 14], and repeated transfer of cells [3, 15, 16]. Other approaches involve the use of an agent which is relatively harmless to the cyanobacteria but is toxic to the other bacterial contaminants. Agents used are phenol [17, 18], sodium hypochlorite [19], detergents [18], sodium sulfide [20], UV or gamma irradiation [21-23], elevated temperature [24, 25], and antibiotics [16, 26, 27].

To explore Spirulina as a possible source of food, feed and important commercial products, we attempted more effective methods for the isolation and purification of Spirulina. This aided the successful production of axenic cultures for our further study.

For commercial purposes, it is especially fruitful and important to isolate mutants or search for newer strains endowed with more favourable characteristics like faster growth, better biomass yield, higher specific cell constituents, temperature adaptability, etc. Thus, a systematic screening of nine different strains available in the germplasm collection was performed to identify the elite strains in terms of biomass and phytochemical production.

RESULTS AND DISCUSSION

Figures 1 A to 0 are phase contrast photomicrographs of the various purified strains of Spirulina from our germplasm collection. **Table 1** gives the list of the strains along with their sources. These strains maintained in stock agar slants were also grown in liquid medium. The composition of the Zarrouk's medium [28] is given in **Table 2** and the procedure for making agar slants is summarized in **Table 3.**

To check the purity of the strains, aliquots were examined critically using phase-contrast and oil immersion microscopy. For additional macroscopic tests, small drops of the liquid culture were placed on plates prepared with Zarrouk's medium supplemented with glucose (0.5%, wt./vol). The plates were then incubated in the dark for 2 weeks at 35°C. The contaminants were easily detected by their growth, color, and other typical appearances (i.e., fungal hyphae). The entire area of the test drop was covered with bacterial or fungal growth if the culture was heavily contaminated. A low degree of contamination revealed in the form of microcolonies on the cyanobacterial growth.

A detailed study was performed to compare the efficacy of various p-lactam antibiotics in reducing the number of contaminating bacteria in Spirulina cultures. In literature, ßlactam antibiotics have been effectively used to prevent contamination of heterotrophic bacteria which are generally associated with cyanobacterial isolates [26, 27]. The rationale behind this is that, the antibiotic would kill the rapidly dividing heterotrophic contaminants and spare the cyanobacteria which do not divide or which divide

Table 1 : Various strains of Spirulina in the germplasm collection

Code	Species	Source
FTCC 01	S. platensis	Isolated from a local pond
FTCC 02	S. platensis	Isolated from a local pond
FTCC 0 3	S. maxima	Isolated from a local pond
FTCC 04	S. laxissima	Isolated from a local pond
FTCC 05	S. platensis	West Germany
FTCC 06	S. platensis	Africa
FTCC 07	S. platensis	Vietnam
FTCC 08	S. platensis	Israel
FTCC 09	S. platensis (fresh water form)	IARI, New Delhi
FTCC 10	*S.* subsalsa Thalotolerant)	Sambar Lake, Rajasthan
FTCC 11	S. lonar	Africa
FTCC 12	*S.* oreriilica	Isolated from a local pond
FTCC 13	S. platensis	Israel
FTCC 14	S. platensis	Japan
FTCC 15	S. m i n o r	Bangalore

Figure I. Photomicrographs of the strains of Spirulina from the germplasm collection (x 670)

A)	FTCC 01	S. platensis
B)	FTCC 02	S. platensis
C)	FTCC 03	S. maxima
D)	FTCC 04	S. laxissima
E)	FTCC 05	S. platensis
F)	FTCC 06	
G)	FTCC 07	S. platensis
H)	FTCC 08	S. platensis
I)	FTCC 09	S. platensis
J)	FTCC 10	S. subsalsa
K)	FTCC 11	S. lonar
L)	FTCC 12	S. oreriilica
M)	FTCC 13	S. platensis
N)	FTCC 1*	S. platensis
O)	FTCC 15	S. minor

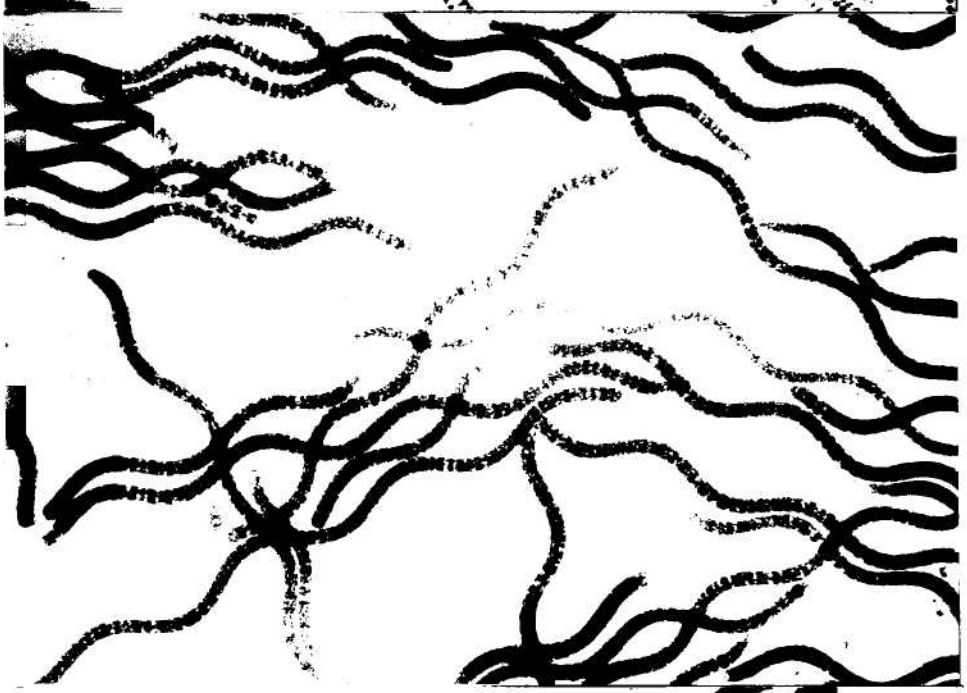
0

FTCC 01 ***S. platens;s***

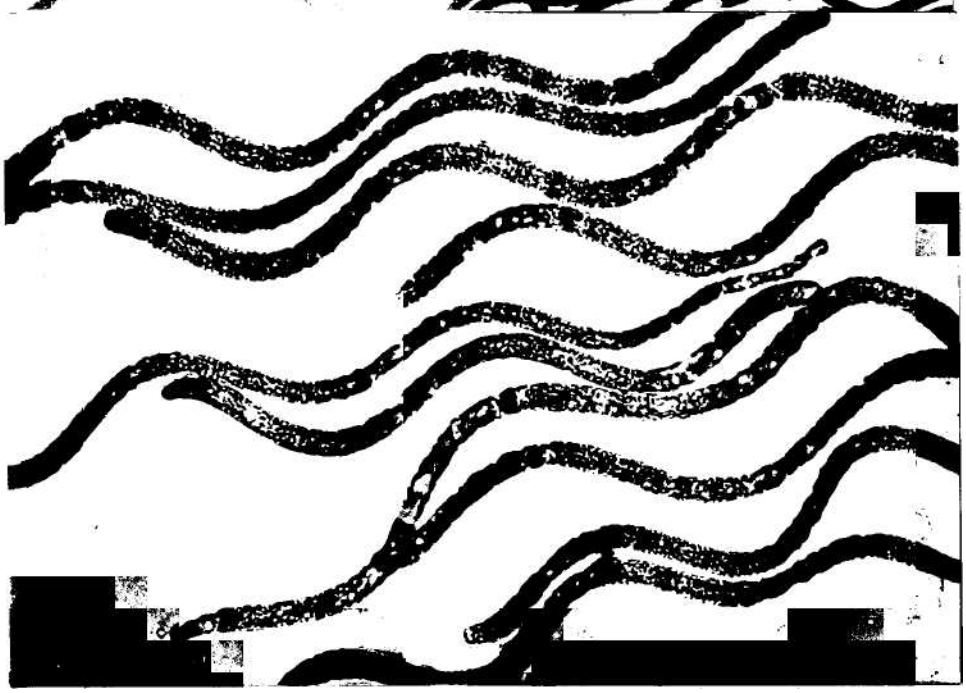
FTCC 02 . ***platensis***

FTCC 03 ***is.maxima***

FTCC 04
S./axissima



FTCC 05
S.platensis



FTCC 06
S.platensIs

Table 2 : Chemical composition of the Zarrouk's medium

Ingredient	Quantity g/L
$NaNO_3$	
NaCl	
K_2SO_4	
K_2HPO_4	
$MgSO_4.7H_2O$	
$FeSO_4.7H_2O$	
$CaCl_2.2H_2O$	
" EDTA	
A_5 solution	
* **AS solution**	
H_3BO_4 ,	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
MoO_3	0.01
$CuSO_4.5H_2O$	0.08

Table 3 : Protocol for maintaining stock cultures of Spirulina 01 agar slants

Parameter	Procedure
Nutrient medium	Zarrouk's medium
Preparation	a) r 16.8 g of NaHCO3 dissolved in 500 ml water b) dissolved in 500 ml water and added 20 g (2%) agar. sterilized separately in an autoclave at 15 lb pressure for 15 min, cooled to 50^{0}C and mixed in equal proportion. The pH was 9-10. Distributed into sterile culture test tubes and slants made.
Method of inoculation	a) 1 drop of Spirulina culture suspension was added directly onto agar slants and spread aseptically. b) Culture from another agar slant was aseptically streaked on the medium in a freshly prepared slant.
Culture maintenance	Inoculated test tubes were incubated at room temperature and illuminated with 40-W cool-white fluorescent lamps at an irradiance of 3 to 5 Klux.
Interval for sub-culturing	20 days interval

slowly. But, this did not solve the contamination problem totally as the B-lactam antibiotics used had a narrow antibacterial spectrum. Imipenem is a relatively new ß-lactam antibiotic which inhibits peptidoglycan biosynthesis and offers a very broad antibacterial spectrum [29, 30]. Hence, imipenem was compared with other ß -lactam antibiotics **(Table** 4), some of which have been previously reported [26, 27].

Five equivalent cultures of contaminated Spirulina platensis (FTCC 06) were supplemented with sterile nutrient solution (SNS), and four of the cultures each received a different antibiotic. Immediately after the additions were made, the number of contaminant bacteria present in each of the cultures was determined. All the cultures were then incubated in dark for 18 hrs, after which the number of bacteria present in each culture was counted again. The results are presented in **Table** 4. With the treatment of *50* μg of imipenem per ml, the number of contaminant bacteria was 0.7% of the initial number. Of the other antibiotics tested, ampicillin and penicillin G were observed to be ineffective in reducing the initial number of bacteria, while cefoxitin, a semisynthetic derivative of cephamycin C [31], was less effective than imipenem. Thus imipenem was found to be the best of all the antibiotics tested **(Table** 4). This was reflected in the observation that after plating, viable cyanobacteria were detected from each of the antibiotic treated cultures while only the imipenem-treated culture produced axenic Spirulina growth.

The greater capacity of imipenem to reduce the number of contaminant bacteria and the ability of cyanobacteria to tolerate incubation in its presence in the dark appear to make imipenem a superior antibiotic to generate axenic cultures of Spirulina.

Figures 2 A & B show the Spirulina cultures maintained in agar slants and liquid medium (shake flask culture), respectively. Earlier studies showed the role of light and temperature in the growth of Spirulina [32-34]. The optimum light intensity for the growth of Spirulina is 100-150 μE m-2 s^{-1} .

Table 4 : Comparison of the efficacy of various antibiotics to reduce the number of contaminant bacteria present in a culture of Spirulina platensis

Antibiotic (50 µg/ml)	Contaminant bacteria/ml culture (x 10) (mean ±SD)	
	Before antibiotic treatment	**After antibiotic treatment**
Imipenem	**0.67 (±O.06)**	0.005 (±0.OOl)
Cefoxitin	**1.1 (±O.03)**	0.10 (±0.OOl)
Ampicillin	**0.97 (±0.07)**	2.5 (±0.08)
Penicillin G	**0.73 (±0.04)**	5.8 **(±1.1)**
None (Control)	**1.03 (±O.06)**	7.8 (±2.4)

Experiments were carried out in triplicates
Standard deviation is given within brackets

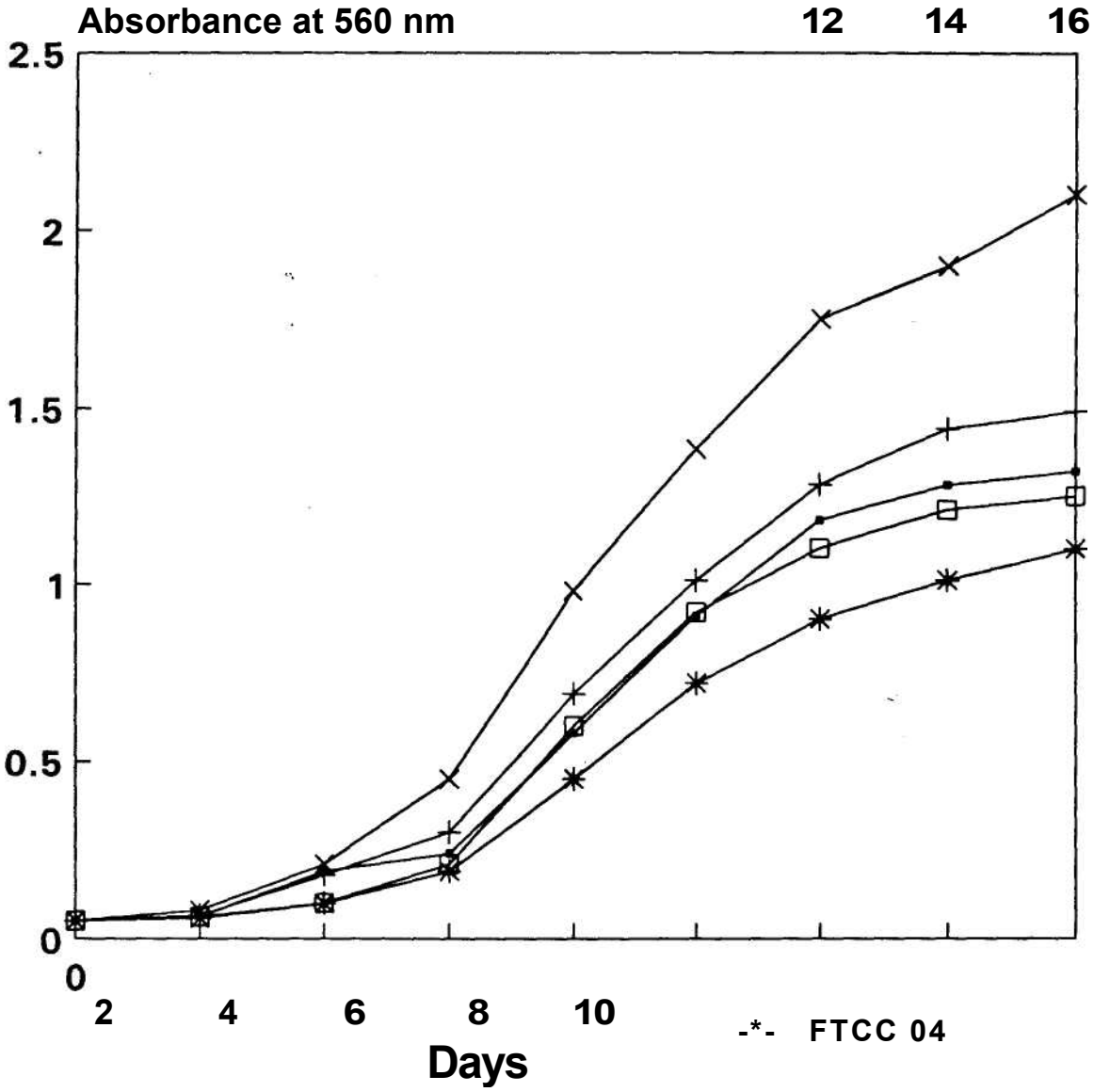
The optimum temperature is between 30° and 40°C, with a mean of about 35°C. The average temperature of 35°C for the optimum production of Spirulina has been reported [32-34]. The temperature maxima of Spirulina are in general higher than those of most eukaryotic algae [24]. Thus, temperature selection offers a means for primary isolation of this alga, particularly from samples where they constitute a small minority of the total algal population. The utility of this technique might wel) be increased by varying other parameters such as light intensity, pH value and nutritional factors.

For the experiments on the evaluation of strains for biomass yield and specific cell constituents, cultures were grown in glass carboys (10 L). Inoculated carboys were kept at room temperature and air was bubbled at the rate of 200 ml/min into each carboy **(Fig.** 2C). The growth was monitored by reading the absorbance at 560 nm on alternate days. **Figures** 3 A&B give the growth curve of the strains screened. **Tables 5 a,b** & **c** give a comparison of nine different strains of Spirulina in terms of growth and production of important phytochemicals. The amount of biomass, ash, protein, pigments (chlorophyll a, phycocyanin and β-carotene), lipids and SOD activity were estimated in those strains **(Tables** 5 **a,b** & c).

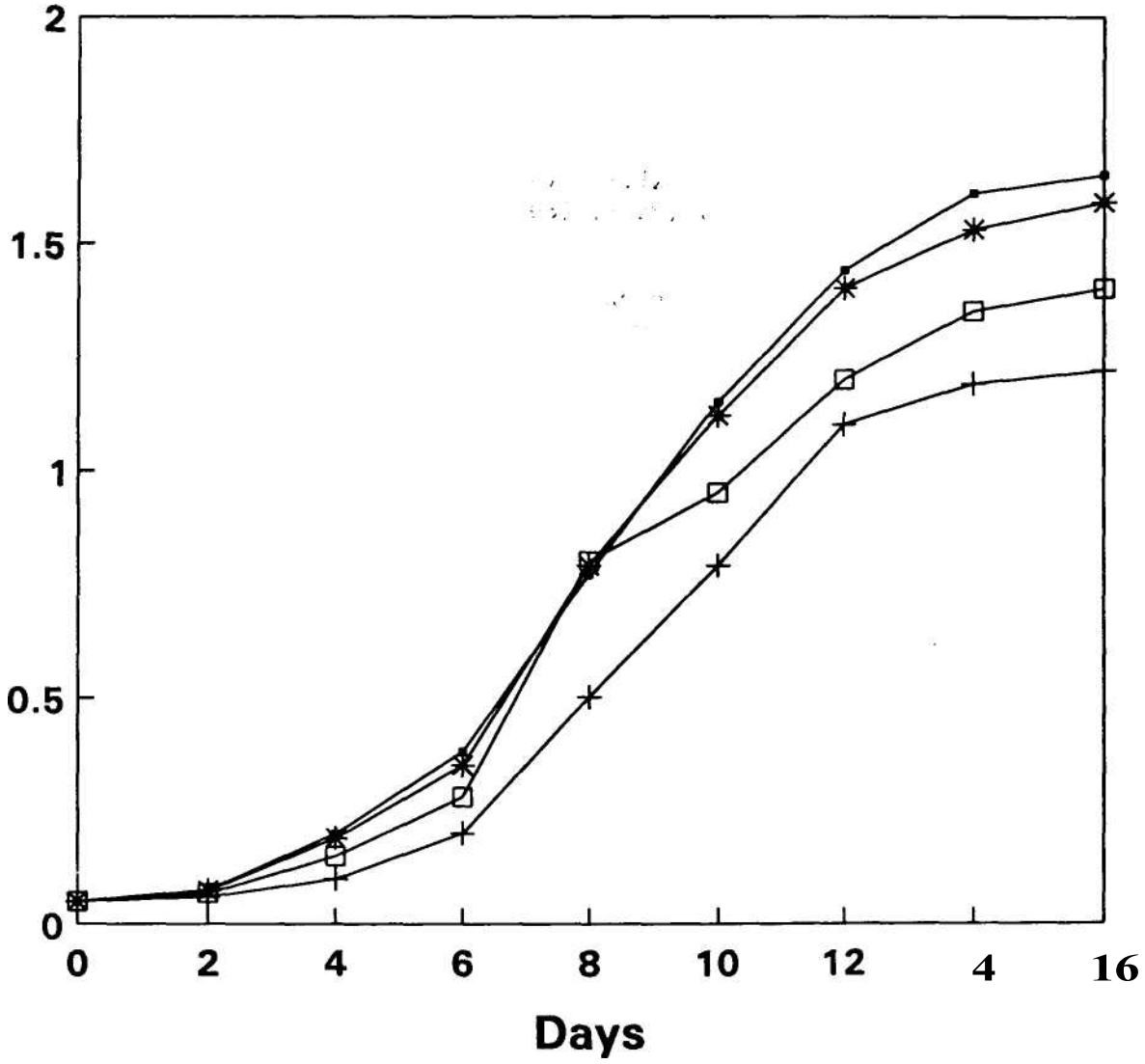
FTCC 06 had the highest content of biomass, exhibiting the fastest growth rate. This was followed by FTCC 07 and FTCC l O in the total biomass yield. FTCC 04 (Spirulina laxissima) was found to be very poor in biomass production. FTCC 06 also had the highest protein content of nearly 62%. The protein content of FTCC 01 (51.1%) and FTCC 03 (48.8%) was fairly good **(Table 5a).**

FTCC 06 had the highest phycocyanin content too. It was nearly 18% of the dry weight. As obseryd earlier for protein, phycocyanin content also was equally good in FTCC Ol and FTCC 03 **(Table 5b).** Thus, FTCC 06 was the best for the production of biomass, protein and phycocyanin among the strains screened.

Figure 3A. Growth 'curve of various strains of Spirulina



38. Growth curve of various strains of Spirulina



Absorbance at 560 nm

- **FTCC09**

FTCC07 -*- **FTCC 13**

FTCC 10

Table 5 : Phytochemicals of different strains of Spirulina (quantified after 10 days of growth)

Phyto-~cal	FTOC 01	FPOC 03	FTOC 04	FTCC 05	FTCC 06	FTOC 07	FTCC 09	FTCC 10	FTOC 13
a) Biomass and important constituents of Spirulina									
Biomass (g OW/L)	1.89 (0.15)	2.15 (0.17)	0.87 (0.09)	1.75 (0.13)	**2.71** (0.17)	2.32 (0.15)	1.74 (0.15)	2.23 (0.16)	1.94 (0.14)
Ash (% OW)	6.10 (1.20)	16.0 (1.08)	19.0 (1.47)	8.07 (1.63)	9.12 (1.25)	9.83 (1.73)	11.0 (1.15)	10.3 (0.94)	8.55 (1.42)
Protein (% OW)	51.1 (1.45)	48.8 (1.20)	42.0 (1.67)	34.2 (1.37)	**62.2** (2.71)	37.6 (2.39)	35.9 (1.84)	38.5 (2.61)	40.1 (2.19)
Lipids (mg/g OW)	42.1 (2.38)	65.8 (3.48)	26.0 (2.26)	45.6 (3.16)	37.2 (2.80)	29.9 (2.28)	53.4 (1.91)	48.2 (3.79)	39.2 (2.54)
b) Pigments **of Spirulina**									
Chl-a (mg/g OW)	12.5 (0.74)	11.2 (0.57)	3.3 (0.44)	8.1 (0.99)	11.8 (0.85)	10.3 (0.50)	9.3 (0.78)	11.3 (0.79)	9.9 (0.67)
Phycocyanin (% OW)	13.3 (1.12)	13.6 (0.94)	6.7 (0.88)	11.2 (1.07)	**18.1** (1.28)	8.3 (0.98)	11.1 (1.49)	13.4 (1.30)	9.1 (1.45)
p-carotene (mg/g OW)	0.77 (0.05)	1.08 (0.08)	0.72 (0.04)	0.75 (0.03)	0.95 (0.04)	0.79 (0.05)	0.52 (0.03)	0.94 (0.06)	**1.40** (0.09)
c) SOD of Spirulina									
SOD (IU/mg protein)	8.2 (O.14)	**8.3** (O.13)	6.3 (0.30)	6.6 (O.12)	8.0 (0.15)	7.5 (O.11)	6.7 (O.12)	8.1 (O.14)	6.9 (0.09)

Data given are averages of three independent experiments.
Standard deviation is given within brackets

Maximum SOD activity was exhibited by FTCC 03 (Spirulina maxima). The SOD activity of FTCC 01, 06 and 10 were also comparable to that of FTCC 03 **(Table 5c). β**-carotene production was more in FTCC 13 than in any other strain. The content was nearly 1.5 times more than that present in FTCC 06 **(Table 5b).**

In view of the beneficial qualities of FTCC 06 like faster growth rate, better biomass, higher protein, and phycocyanin production, it was identified as the most suitable strain for commercial exploitation. This is presently being used for the technology development of large scale cultivation and down stream processing of Spirulina at CFTRI, Mysore. This also is currently used for large scale extraction of phycocyanin for industrial purposes. With a view to improve the nutritional and commercial value of this strain (FTCC 06), further experiments on the enhancement of p-carotene and phycocyanin levels were carried out. The principal carotenoids of this strain were also identified (Chapters 2 and 3).

Viable protoplasts in good yield were successfully prepared from the same strain (FTCC 06), by employing two different methods. The protoplast regeneration was demonstrated. Also, attempts were made to genetically transform the intact trichomes and protoplasts of this strain by direct DNA uptake (Chapter 5).

Since FTCC 03 exhibited the highest SOD activity, this strain was used for the study on purification and characterization of SOD followed by the antioxidant properties of the purified SOD (Chapter 4).

EXPERIMENTAL

Chemicals used

Deionized and double distilled water was used throughout. Aqueous stock solutions (0.5% [wt./vol]) of cefoxitin, ampicillin, penicillin G, imipenem, and cycloheximide (1.25% [wt./voID were prepared fresh, sterilized by

filtration, and added to cooled molten agar or broth media. Stock solutions of nystatin (10%[wt./vol]) were prepared in N,N-dimethylformamide. Penicillin G (potassium salt), cefoxitin, imipenem, cycloheximide and nystatin were purchased from Himedia Laboratories and Pvt. Ltd. Ampicillin (sodium salt) was from Sigma Chemicals and Co. All other chemicals used were of the highest purity avairable locally.

Germplasm collection

Cultures of 15 different strains of Spirulina obtained from India and other countries were purified and maintained in Zarrouk's medium either in agar slants **(Fig.** 2A) or in liquid medium **(Fig.** 28). The cutlures were subcultured once in 20 days.

Growth medium

The composition of the Zarrouk's medium is given in **Table 2** and the procedure for making agar slants is summarized in **Table 3.**

Growth conditions

The broth cultures were grown at a light intensity of 100-150 } μE m-2 s-l , a temperature of 35°C and an agitation of 90 rprn on a rotary shaker. The slants were incubated at room temperature and illuminated with 40-W cool-white fluorescent lamps at a light intensity of 3 to 5 Klux.

Purification and development of axenic cultures with antibiotics

The method described by Ferris and Hirsh (1991) was modified and used [35].

To the cultures with sufficient biomass (0.0. at 560 nm - 1.2), *400* }l1 of a sterile nutrient solution (SNS) consisting of 2.5% (wt./vol) sucrose, *0.5%* (wt./vol) yeast extract, and 0.5% (wt./vol) bacto-peptone were added

along with 400 µl of sterile antibiotic solution **(Table** 4) to give a final antibiotic concentration of 50 } µg/ml. The cultures then were incubated for 18 to *24* hrs in the dark at 180-200 rpm. After incubation, the cyanobacteria were harvested by centrifugation at 17,000 x g for 15 min at 25°C. The cells were washed twice by centrifugation with volumes of sterile Zarrouk's medium equal to the original culture volume and finally suspended in 1/10 of the original volume. The cyanobacterial cells were gently dispersed with a sterile tissue homogenizer in order to produce a cell suspension which could be easily pipetted and plated onto Zarrouk's agar medium containing nystatin and cycloheximide. The plates were incubated for 2 to *4* weeks and observed at weekly intervals for the growth of Spirulina. With a dissecting microscope (x10 to x50 magnification), purified filaments of cyanobacteria were picked and transferred to plates of nutrient medium. The purity of the isolates was confirmed by phase-.contrast microscope (x500 to x l,250 magnification) and by inoculating Spirulina cells into nutrient medium supplemented with 0.01% (wt./vol) (each) glucose, yeast extract and bacto-peptone. Cultures were incubated at 25°C with shaking at 180-200 rpm under ambient atmosphere and under anaerobic conditions. If no growth of heterotrophic bacteria was observed under this condition after incubation for 1 month, the cultures were judged axenic.

Screening of different strains

A screening of nine different strains of Spirulina viz., Spirulina platensis (FTCC 01, FTCC 05, FTCC 06, FTCC 07, FTCC 09 and FTCC 13), Spirulina maxima (FTCC 03), Spirulina laxissima (FTCC 04) and Spirulina subsalsa (FTCC 10) was carried out for biomass and phytochemicals.

Estimation of biomass

Growth was monitored by following the optical density of the cells at 560 nm in a Shimadzu Spectrophotometer periodically which was converted to dry weight by a standard graph of optical density (A560) VS. dry weight. Biomass was determined on the 10th day of growth and expressed as dry weight per litre.

Quantification of cellular constituents

Proteins were quantitated using Kjeldahl's method and chlorophyll-a by the method described in AOAC Official Methods of Analysis (1984).

Lipids [36]: The cells were harvested and pelleted by centrifugation (I,OOOxg, 10 min). The tubes containing the pellet were placed on ice and added 10 ml of ice cold 0.2 N $HClO_4$. Thoroughly vortexed to suspend the cells. After 15 min at 4°C, samples were centrifuged at 4°c (5,000xg, 10 min) and the supernatant removed. Added 10 ml of chloroform-methanol (2:1 v/v) to the pellet, resuspended the cells, allowed to stand for 5 min at room temperature. The samples were centrifuged (5,000xg, 10 min) and the supernatant coliected.

Added 0.2 volumes of water to the combined chloroform-methanol extracts of the cells. Shook for 5 min to mix and centrifuged to separate the phases. The organic phase (lower) was collected, leaving behind a precipitate that formed at the interphase. The upper aqueous phase was discarded. The solvent extract was evaporated under a stream of nitrogen to a final volume of 2 mI.

Known volumes of samples and standards were transferred to tubes, evaporated to dryness under vacuum and added 2 ml of dichromate solution (2.5 g of $K2C^{r}2^{0}7$ *in* 1L °f conc. H2SO4). Placed in boiling water bath for 45 min. Cooled the tubes, removed 1 ml from each sample, diluted to 100 ml with water and read A350. The assay is based on the reduction in absorbance at 350 nm as the dichromate is reduced by increasi'\tamounts of lipids.

p-carotene [37] (details given in Chapter 2), phycocyanin [37] (details in Chapter 3) and SOD activity [38] (details in Chapter 4) were also quantified.

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CHAPTER. 2

CAROTENOIDS OF *SPIRULINA.*

SUMMARY

The carotenoid composition of Spirulina platensis (FTCC 06) was analyzed. The total carotenoid content of this species was found to be 3.51 mg/g OW. Eleven compounds were identified: neoxanthin, violaxanthin, canthaxanthin, echinenone, myxoxanthophyll, zeaxanthin, lutein, phytofluene, phytoene, β-cryptoxanthin and β-carotene. Among these, neoxanthin, violaxanthin, lutein, phytofluene and phytoene are reported for the first time as the carotenoids of the order Nostacales.

Enhancement in the levels of β-carotene in Spirulina E.latensis in response to variations in light intensity, sulphate and/or nitrate stress and UV irradiation is demonstrated. Cells grown at a light intensity of 150 μ*E* m-2 s-1 was found to be optimal for J3-carotene production. On reducing the amount of nitrate and sulphate supplied in the growth medium by 50%, nearly 1.5 fold increase in β-carotene content could be achieved with only 1096 reduction in growth rate. The cells selected after UV treatment produced 3 times higher levels of p-carotene than the wild type cells. But the mutants were not found to be stable.

The cyanobacterial extracts containing carotenes and various carotenoids are frequently used as natural colouring materials. They possess antioxidant activity, especially in the presence of light [1]. Carotenoids also have important metabolic functions in animals and man, including conversion to Vit A, enhancement of the immune response and protection against diseases such as cancer by way of scavenging free radicals [2, 3]. β-carotene, a major carotenoid of Spirulina is a potential antioxidant having anti-carcinogenic *[4,* 5] and radio-protective effects. Spirulina contains upto 2,000 IU/g dry weight of β-carotene [6].

Since Spirulina is being cultivated as ' Health Food' in several countries [7], the natural products of nutritional and commercial importance isolated from Spirulina have been receiving great attention. The purification and identification of the principal carotenoids of Spirulina platensis (FTCC 06) is described here. Five of the carotenoids were detected for the first time as natural products of this alga.

Based on reports on the enhancement of β-carotene levels in Dunaliella [8, 9], attempts were made in our study to increase β-carotene production in Spirulina platensis (FTCC 06), a strain which was identified here as an elite strain in terms of biomass and specific cell constituents. This study aims at increasing the nutritional and commercial value of this strain which is being cultivated in a large scale at Central Food Technological Research Institute, Mysore.

RESULTS AND DISCUSSION

Principal carotenoids of Spirulina

The total carotenoid content of Spirulina platensis was found to be 3.51 mg/g DW. Most of the carotenoids present in the extract of this species were identified : neoxanthin (I), violaxanthin (II), canthaxanthin (III), echinenone (IV), myxoxanthophyll (V), zeaxanthin (VI), lutein (VII),

phytofluene (VIII), phytoene (IX), p-cryptoxanthin (X) and p-carotene (XI). Figure 1 shows the identification of the carotenoids by comparison with authentic reference compounds (peaks III, V, VI, X, XI) and tentative identification with reported spectral properties (peaks I, II, IV, VII, VIII, IX)' [10]. The spectral properties of the identified principal carotenoids are listed in Table 1.

The carotenoids β-carotene, β-cryptoxanthin, zeaxanthin, echinenone, 4-k.eto-3'-hydroxy β-carotene, myxoxanthophyll, canthaxanthin and 4-keto-3-hydroxy β-carotene are already reported in Spirulina platensis [11]. To the best of our knowledge, compounds neoxanthin (II), violaxanthin(II), lutein(VII), phytofluene (VIII) and phytoene (I X) are detected for the first time in this alga.

The quantitative distribution of carotenoids in blue-green algae can vary with cultural conditions. Myxoxanthophyll was found *to* be the major pigment constituting nearly 32% of the total carotenoids in Spirulina (Table 2). This was followed by β-carotene (29%) and zeaxanthin (14%) in abundance. According to the observations made here, blue-green algae can produce epoxycarotenoids since neoxanthin and violaxanthin are detected in Spirulina. These have been reported so far only in green algae, for eg_o Dunaliella spp. [12]. Eventhough traces of lycopene and β-carotene which are components of the carotenoid biosynthetic pathway in cyanobacteria are shown to be present in a Cyanophyte, there is no report regarding the occurrence of phytofluene and phytoene in cyanobacteria which are also intermediates in the same pathway. In our study, both phytofluene and phytoene were found to occur as the carotenoids of Spirulina. Lutein also was detected here although carotenoids with E-rings probably do not exist in blue-green algae [13].

The characteristic carotenoids common to the blue-green algae are β -carotene [14], echinenone [15, 16], zeaxanthin [14, 16] and myxoxanthophyll [14, 16]. β-carotene is normally the only carotene reported [14] and blue-green algae apparently cannot make E-end groups [13]. However, traces of t-carotene and lycopene have been found in a hot spring Cyanophyte, a species

Figure 1. HPLC profile of the carotenoids of Spirulina

Peak 1 Neoxanthin
2 Violaxanthin
3 Canthaxanthin
Echinenone
5 Myxoxanthophyll
6 Zeaxanthin
7 Lutein
8 Phytofluene
9 Phytoene
10 β -cryptoxanthin
11 β-carotene

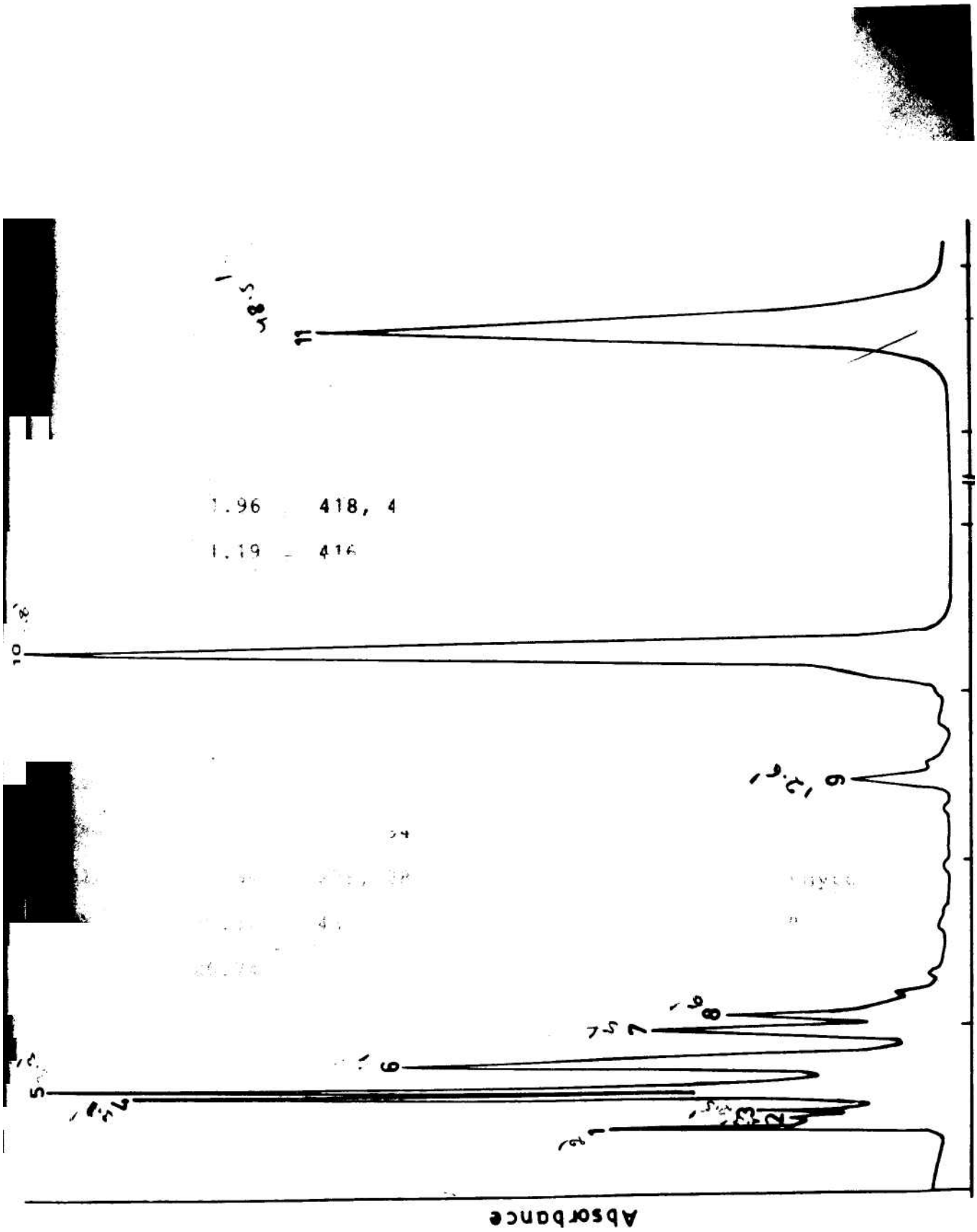

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Table
1 : Identification platensis of principalcarotenoids **of Spirulina platensis**

Peak No.	**Relative % of carotenoids (at 450 nm)**	**Unknown max(nm)**	Ref. compound max(nm)	**Identification**
I	1.96	**418**, , 442, 471	419, 443 471	Neoxanthin
II	1 . 19	**416**, , 440, 469	415, 440 469	Violaxanthin
III	1.21	477	477	Canthaxanthin
IV	13.49	463	464	Echinenone
V	19.29	449, , 472, 505	449, 475 505	Myxoxanthophyl
VI	8.70	422, , 450, 480	422, 450 481	Zeaxanthin
VII	3.51	418, , 445, 474	418, 444 474	Lutein
VIII	2.84	331, , 347, 367	331, 347 367	Phytofluene
IX	2.84	278, , 287, 298	278, 287 299	Phytoene
X	20.32	430, , 452, 475	431, 452 478	p-ryptoxanthir
XI	26.74	428, , 451, 477	428, 451 476	β -Carotene

* The values correspond to the area of peaks shown in Figure 1.

Table 2 : **Quantitative distribution of carotenoids in Spirulina platensis**

Carotenoid	**Amount (mg/g DW)**
Neoxanthin	**NQ**
canthaxanthin	**NQ**
Canthaxanthin	**0.04**
Echinenone	**NQ**
Myxoxanthophyll	**1.06**
Zeaxanthin	0.47
Lutein	**NQ**
Phytofluene	**NQ**
Phytoene	**NQ**
B-cryptoxanthin	**0.17**
β-carotene	0.95

Quantified using standard compounds
Q - not quantified due to the non-availability of authentic standards

of Oscillatoria and p-zeacarotene in a laboratory culture of Spirulina Qlatensis [13]. Mutachrome (5,8 - epoxy p-carotene) is occasionally encountered (e.g. in Ocillatoria agardii) *[17]* as is isocryptoxanthin [18]. Recent work has indicated that the diketo carotenoid canthaxanthin is also quite widely distributed [19, 20]. The previously reported pigment aphanicin ha~ now been identified as canthaxanthin [16]. The keto carotenoid echinenone is unique in normal' photosynthetic cells of blue-green algae [13], whilst myxoxanthophyll is a carotenoid glycoside which is present in all blue-green algae so far examined except Phormidium spp. [20].

The algal extracts containing carotenoid pigments are widely used as antioxidants. The antioxidative activities of pigments from dried marine algae has been reported [21]. According to this report, pigments extracted from 6 dried marine algae (1 green, 3 brown, 2 red) had antioxidant properties. Another report shows the antioxidative effect of the algal extracts of seven species of marine algae on the preservation of sunflower oil [22].

The carotenoids act by quenching singlet oxygen produced during photosynthesis [23], owing to their multiple conjugated double-bond system. The presence of carotenoids in edible oils may also help protect against the formation of singlet oxygen by blocking light transmission through the oil. Canthaxanthin, astaxanthin and the other carotenoids containing oxo groups at the 4(4)-position in p-ionone ring system can serve as more effective antioxidants than p-carotene in peroxyl radical-dependent lipid peroxidation. Thus the xanthopylls may be of importance as biological antioxidants *[24]*.

Enhancement in β-carotene levels

Based on the studies on p-carotene enhancement levels by induction in Dunaliella [8, 9], Spirulina was subjected to different growth conditions and controlled UV irradiation to obtain higher levels of β-carotene.

In the experiments conducted to increase the cellular constituents by varying the light intensity required for growth **(Fig.** 2), there was reduction in growth of about 29% in cultures grown under low light intensity (50 PE m^{-2} s^{-1}) as compared to cultures grown at a light intensity of 150,UE m^{-2} s^{-1}. In the case of cells grown at high light intensity (300 ,uE m^{-2} s^{-1}), the cells started bleaching after 5 days as indicated by their chlorophyll-a content (8.28 mg/g OW) which was relatively lower than 13.68 mg/g OW and 11.31 mg/g OW in cells grown at 50 ,uE m^{-2} s^{-1} and 150 ,uE m-2 s-1, repectively. The accumulation of total carotenoids was 6% higher in cells grown at low light intensity and it was nearly 15% less in cells grown at high light intensity as compared to the cells grown at 150 µE m-2 s-1 . Eventhough the total carotenoids was more in cells grown at low light intensity, the percentage of p-carotene was not higher enough than the cells growing at 150µE m-2 s-1 , thus giving a slightly lesser β-carotene yield (0.82 mg/g OW) in those cells. Therefore, the light intensity of 150 µE m-2 s-2 was found to be optimal for β-carotene accumulation.

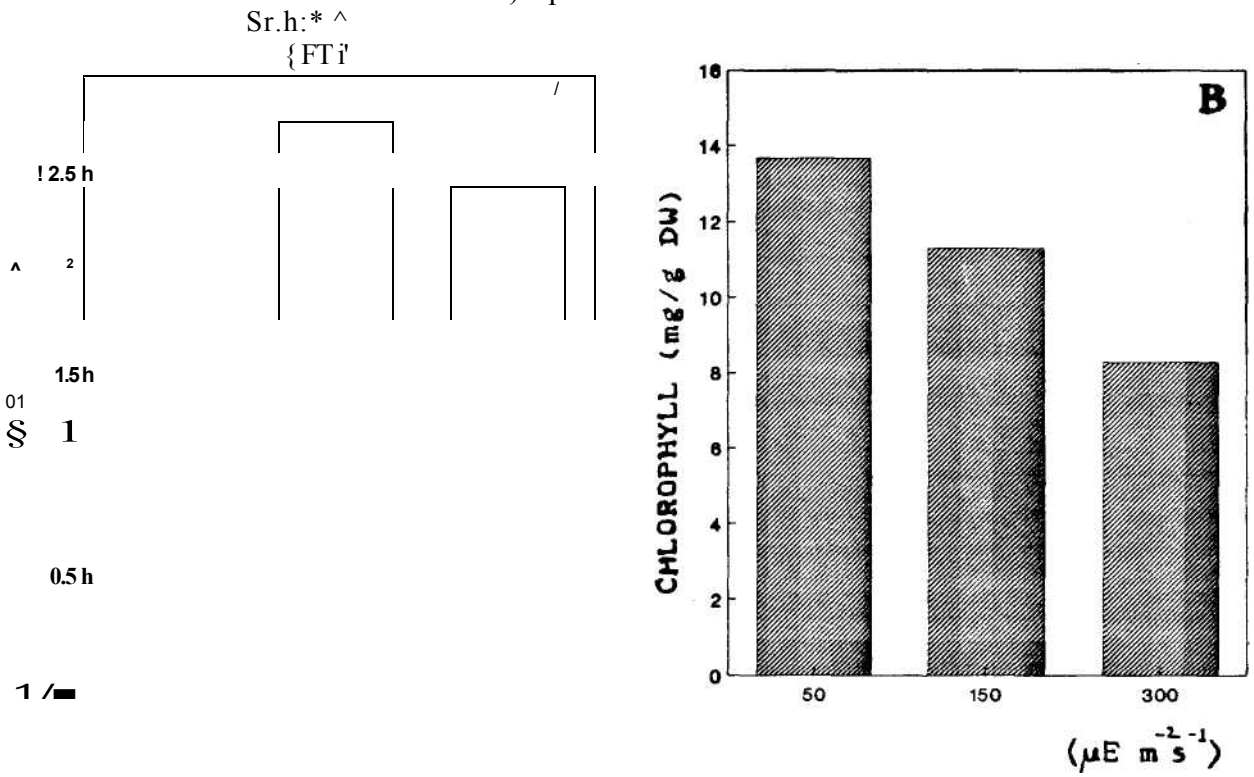
When sulphate was reduced by 75% ($Mg504.7H_20$- 0.05 g/L; $Fe504.7H_20$- 0.0025 g/L.; K_2504- 0.25 g/L) with or without nitrate stress, the β-carotene content was 49-56% lower than the control **(Table 3).** This reduction was not observed when nitrate was decreased by *75%* (NaNO3- 0.625 g/L). The concentration of the sulphate and nitrate, when lowered by 50%, an increase in p-carotene content from 0.94 to 1.39 mg/g OW was obtained, which is nearly 1.5 fold increase in those cells. The stress condition (50% reduction in 5°4 and NO3 strengths) resulted in only 10% reduction in biomass **(Table 3).** Unlike in Ounaliella bardawii where there is over production of p-carotene by photo-induced activation of genes [8], there was no change in j-carotene by high light intensity. Also, a light mediated change in enzyme activity, which was shown as the cause for photo-induced carotene formation in Chlorella mutants and in Euglena [25] was ruled out in the case of Spirulina.

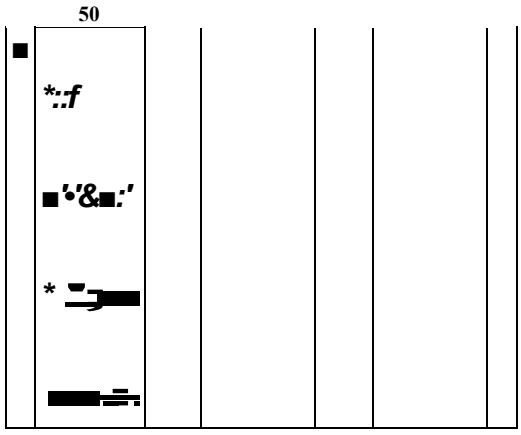
High β-carotene producing cells

When the cells were subjected to mutagenesis by UV irradiation to select mutants producing high p-carotene, there was only 5% survival with the dosage

Figure 2. The effect of light intensity on

A) Biomass
B) Chlorophyll-a
C) Total carotenoids
D) p-carotene





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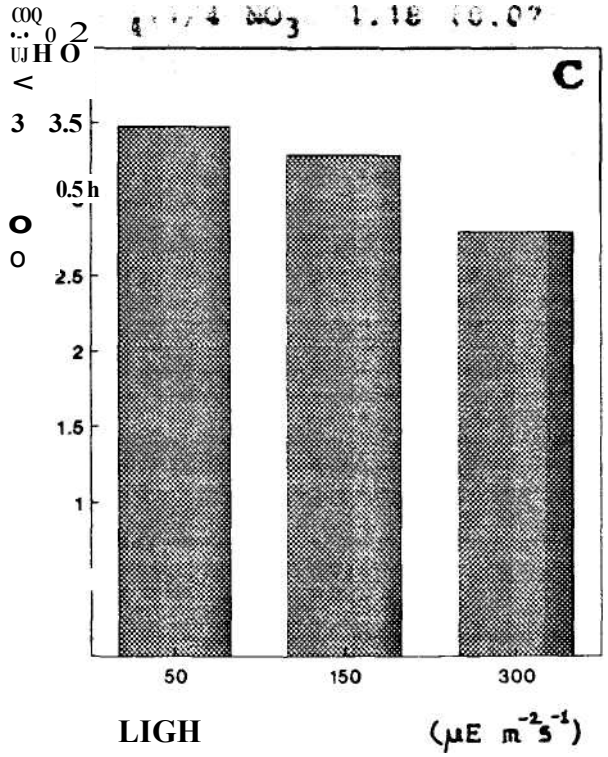

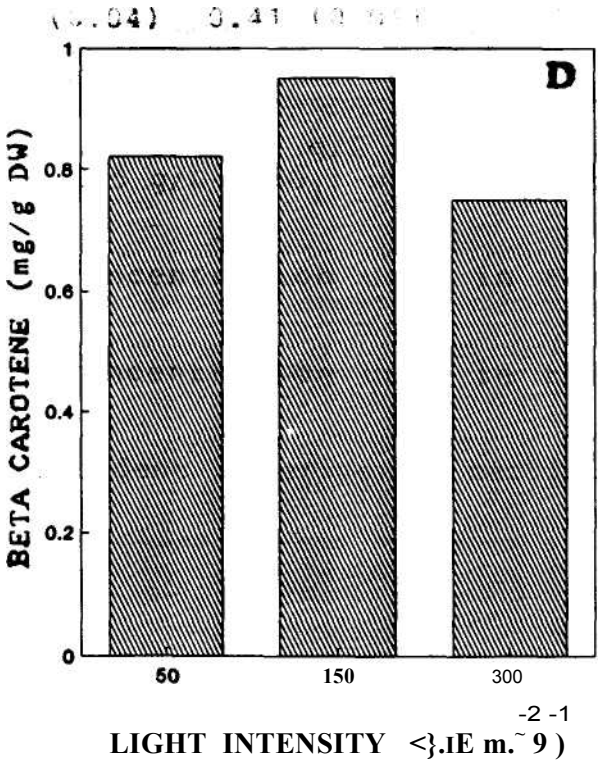

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Table 3 : Enhancement of p-carotene in Spirulina platensis (FTCC 06) under sulphate and nitrate stress

Stress condition	Biomass yield (g OW/L)	Total carotenoids (mg/g OW)	p-carotene (mg/g OW)	% incre
FS SO_4+FS NO_3 (Control)	2.72 (0.05)	3.29 (0.04)	0.94 (0.04)	10
1/2 SO_4+FS N03	2.58 (0.05)	2.79 (0.03)	1.05 (0.04)	111.7
1/4 SO_4+F8 N03	2.06 (0.02)	2.33 (0.04)	0.42 (0.01)	44.7
1/2 N03+F8 SO_4	2.52 (0.03)	3.09 (0.06)	1.12 (0.02)	119.1
1/4 N03+FS 8°$_4$	2.09 (0.04)	3.31 (0.06)	1.13 (0.02)	120.1
1/2 SO_4+1/2 NO_3	2.43 (0.02)	3.05 (0.05)	1.39 (0.03)	147.9
1/2 8°$_4$+1/4 N03	1.87 (0.04)	3.03 (0.04)	1.00 (0.02)	106.4
1/4 SO_4+1/2 NO_3	1.93 (0.03)	2.76 (0.03)	0.48 (0.01)	51.1
1/4 SO_4+1/4 NO_3	1.18 (0.02)	2.59 (0.04)	0.41 (0.01)	43.6

FS SO_4/NO_3 - Full strength sulphate/nitrate (0.2 g/L $MgSO_4.7H_2O$ 0.01 g/L $FeSO_4.7H_2O$ 1.0 g/L K_2SO_4 / 2.5 g/L $NaNO_3$) which serve as the control

1/2 SO_4/NO_3 - 50% of the SO_4/NO_3 concentration used in the control

1/4 SO_4/NO_3 - 25% of the SO_4/NO_3 concentration used in the control

Results are averages of three independent experiments carried out in triplicates (~8D)

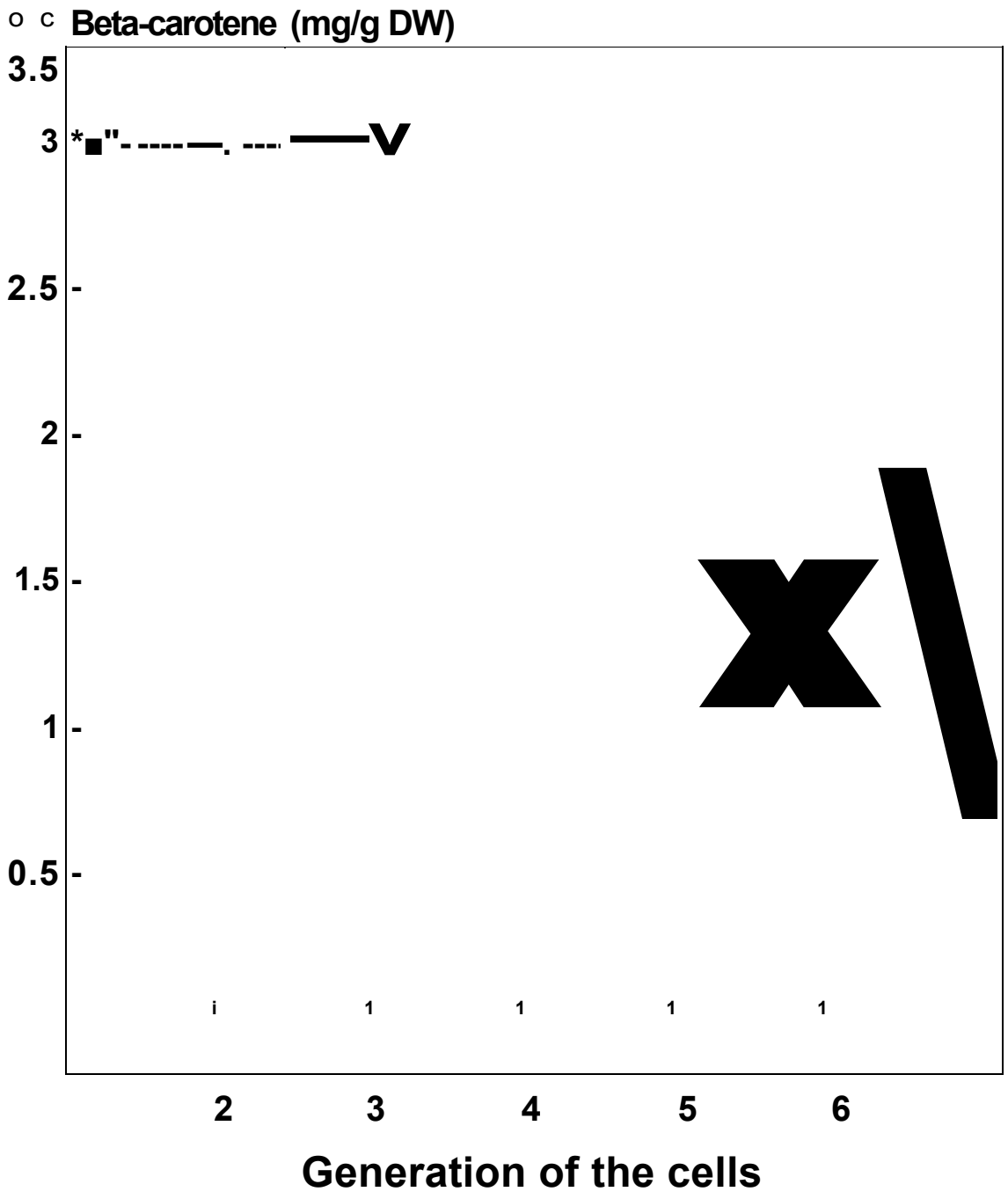
of UV irradiation given. After the selection procedure was repeated thrice as given in the Experimental, the surviving algae grown for 10 days had a β-carotene content of 2.91 mg/g DW which is nearly 3 times more th~n the wild type cells.

These mutant cells were subcultured and maintained under normal growth conditions. The high β-carotene production could be monitored in the cells only for 3 generations after which it started reducing gradually (Fig. 3). The β -carotene content was equal to that of the wild type cells in the 7th generation which showed that the mutants were not stable and the cells reverted back due to photoreactivation. The other possibility is that mutagenesis of filamentous algae poses a special problem in that the colony forming unit is a filament, whereas the target of mutagenesis is a chromosome in a single cell. This means that the resulting colony following mutagenesis can be a mixture of mutant and wild type cells.

The market value of natural β -carotene which is used as pro-vitamin A is > $ 500/Kg and that which is used as food colour is > $300/Kg [26]. Spirulina being a traditional food source in several countries, the 1.5 fold increase in β -carotene content obtained by nutrient stress and 3 fold increase obtained by mutant selection here are significant in terms of the nutritional value of Spirulina rather than its commercial value. For commercial purposes, Dunaliella is a better source of p-carotene where the content is as high as 10% of the cell dry weight [26].

Since the first report on the role of Vit.A in cancer prevention [27], β -carotene rich diets have assumed importance in cancer prevention [28]. Epidemiological evidence is sufficient to suggest that foods rich in carotenes or Vit_o A are associated with a reduced risk of cancer [29]. Besides cancer prevention, β -carotene from Spirulina is of immense value in combating deficiency conditions and general health status. Gopalan (1990) [30] and Narasinga Rao (1991) [31] have emphasized the increased effect of β -carotene from natural sources over that of Vit_o A as a pure substance.

Figure 3. The p-carotene content of the UV-irradiated Spirulina cells in the subsequent generations, indicating the gradual reduction in J3-carotene levels



The mutant selection by UV irradiation was attempted to explore the possibilities of obtaining an over production of β-carotene by this method as reported in Dunaliella. This would probably increase the commercial value of Spirulina as a potential source of p-carotene to compete with Dunaliella. Since the mutant cells could yield an amount which is only 3 times more than the wild type cells and also that the mutants are not stable, the large scale production of β -carotene from Spirulina may not be an immediate possibility.

EXPERIMENTAL

Chemicals used

The standards canthaxanthin, myxoxanthophyll, zeaxanthin and 1-cryptoxanthin were obtained from Hoffman La-roche, Switzerland. β -carotene was from Sigma Chemicals Co., USA. All the solvents used were of HPLC-grade and the other chemicals utilized for the study were of the highest purity available locally. Deionized and triple distilled water was used throughout.

Identification of principal carotenoids

Organism and culture conditions

Clonal and axenic culture of Spirulina platensis (FTCC 06) was used for this study. The culture was grown in Zarrouk's liquid medium [32] in 1 L shake flasks, at a light intensity of 100-150 y and an agitation of 90 rpm on a rotary shaker. m^{-2} s^{-1}, temperature of 32°C

Extraction and estimation of carotenoids

Algal cells from a sample of about 2 ml of liquid culture growing at a logarithmic phase (A560-0.8 approx.) were centrifuged at 1,000 x g for 5 min. The pellet was extracted with methanol in mortar. The extract was separated by centrifugation (1,000 x g, *5* min) and stored at *4°C.* The residual material

was broken down by the pestle to a fine paste and extraction repeated until no more coloured material could be extracted [33]. The extracts were pooled and the total amount of carotenoids estimated as follows.

$$C = \frac{A_{450} \times V \times f \times 10}{2{,}500} \quad [34]$$

where C is the total carotenoids in mg/ml, V is the volume of the extract, f is the dilution factor, and 2,500 is the extinction coefficient. The carotenoid content was expressed in terms of mg/g dry weight. The methanol extract was concentrated for High Performance Liquid Chromatography (HPLC).

Isolation and identification of carotenoids

The separation of carotenoids was carried out on HPLC using a 150 x 4.6 mm C18 Reverse phase column (5 μm spherisorb ODS). The solvent system consisted of methanol:acetonitrile (90:10, v/v) and the flow rate was 1 ml/min. The separated peaks were collected for monitoring the absorption spectra.

The peaks were identified by comparing the retention time as well as the absorption spectra of the unknown with that of the corresponding authentic compounds. In the absence of the authentic standards, the absorption spectra of the peaks were matched with their probable authentic equivalents, with literature data as a guideline.

The identified compounds were quantified using the respective standards by measuring the absorbance at their respective absorption maxima (as given in **Table 1).**

Enhancement of β-carotene levels

β-Carotene Extraction and Quantification

For β -carotene analysis, algal cells from a sample of about 2 ml of liquid culture growing at a logarithmic phase (A560- 0.8 approx.) were centrifuged at 1,000 x g for 5 min and the pellet extracted with 3 ml of ethanol:hexane (2:1, v/v). Two ml of distilled water and 4 ml of hexane were added, thoroughly mixed and centrifuged at 1.000 x g for 5 min-w separate the hexane layer [35]. A preliminary separation of the major carotenoids was done by subjecting this crude extract to Thin Layer Chromatography (TLC). The concentrated extract was applied as a strip on a TLC plate (Silica G, 250 *ym* thick, 20x20 cm) and developed with hexane:acetone (4:1, v/v). The yellow coloured band of interest was immediately scraped off the plate in dark and the carotenoids eluted in dark with diethyl ether. The solvent was evaporated under nitrogen and the residue was dissolved in a mixture of methanol:dichloromethane (4:1, v/v) for HPLC.

The separation of β -carotene was done on HPLC using a 150 x 4.6 mm C18 reverse phase column (5 pm spherisorb ODS). The solvent system consisted of acetonitrile: methanol: dichloromethane (41:50:9, v/v) and the flow rate was 1 ml/min.

The quantification was carried out using β -carotene standard. The retention time of p-carotene was found to be approximately 12 min.

Enhancement of β -Carotene

To study the influence of light intensity on p-carotene production, cells pre-grown (A560 - 0.8 approx.) in low light intensity *(50*)μE m s-1) were centrifuged and resuspended in fresh growth medium and incubated at different light intensities namely 50, 150 and 300) μm-2 s-l . Cultures were analyzed for chlorophyll [36], carotenoids and β-carotene content on the 10th day of incubation.

For nutrient stress experiments to increase p-carotene level, the cells pre-grown as above were resuspended in medium with varying concentrations of nitrate and/or sulphate (as given in **Table 3)** and grown at a light intensity of 150 μE m-2 s-l . β-carotene was estimated after 10 days of incubation.

Selection of high β -carotene producing cells

The cells pre-grown (A560 - 0.8 approx.) in low light intensity (50 μ*E* m-2 s-1) were centrif uged and resuspended in fresh growth medium and incubated at a light intensity of 150M μE m s-1 for 24 hrs. The cells were exposed to mutagenesis condition by UV irradiation (0.68 J/sec) for 30 min and kept in the dark for *24* hrs to prevent photoactivation repair. The algae were then incubated under low light (50 μE m^{-2} s^{-1}) for 6 days. Later, they were exposed to high intensity blue light (300 μE m-2 s^{-1}) for 10 hrs, to select mutants that contain higher β-carotene levels. The cells were allowed to recover under normal growth conditions for 3 days. This selection procedure was repeated thrice. The surviving algae were plated on agar plates andJ3-carotene estimated after 10 days.

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CHAPTER 3

BILIPROTEINS FROM *SPIRULINA*

SUMMARY

A method was developed for the rapid isolation of phycobilisomes using a cationic detergent, cetyltrimethylammonium bromide (CTAB), followed by its characterization. The intact phycobilisomes were released from cells in the presence of CTAB within only 30 sec, thereby reducing the chance of proteolysis of the component phycobiliproteins. The phycobilisome preparation had very little chlorophyll 'contamination. The structural and functional properties were retained as evidenced by spectroscopy and sodium dodecyl sulfate polyacrylamide gel analysis.

Enhancement in the levels of phycocyanin in Spirulina platensis, in response to nitrate stress is demonstrated. When the nitrate concentration in the medium was increased to 2, 3 and 4 times its original strength, the phycocyanin level was enhanced by nearly 8, 18 and 27% respectively. Phycocyanin is a major phycobiliprotein of Spirulina having a wide commercial application.

In the eukaryotic red algae and prokaryotic cyanobacteria, the outer surface of the thylakoid or photosynthetic membrahes is covered by ,rows of closely spaced granular structures. These structures were first postulated to represent an ordered array of the pigmented phycobiliproteins that are associated with the thylakoid membranes. They harvest light energy for photosynthesis in the reaction centres embedded in the thylakoid membranes [1]. In a series of studies, Gantt and Conti [1-3] demonstrated that these granular structures are composed of phycobiliproteins, and they named them phycobilisomes (PBS). Phycobiliproteins with covalently linked bile pigment chromophores may comprise more than 60% of the total soluble cellular protein, or about 20% of the total dry weight of one alga [4, 5].

Phycobilisomes consist of allophycocyanin cores surrounded by phycocyanin and phycoerythrin on the periphery. Phycocyanin is the major constituent of the phycobilisome, while allophycocyanin functions as the bridging pigment between phycobilisomes and the photosynthetic lamellae. In addition to phycobiliproteins, phycobilisomes also contain several colourless polypeptides which are responsible for the molecular assembly of the phycobilisome. Consequently, these polypeptides have been termed linker polypeptides [6].

The colors of the phycobiliproteins arise from the presence of covalently attached prosthetic groups - bilins [7]. The bilins are linear tetrapyrroles derived biosynthetically from heme via biliverdin [8]. The four different isomeric bilins, those found in the phycobiliproteins of cyanobacteria and red algae are phycocyanobilin, phycobiliviolin, phycoerythrobilin and phycourobilin [9]. Several additional tetrapyrroles have been found in cryptomonad phycobiliproteins [10, 11].

The phycobiliproteins were introduced as a novel class of fluorescent tags in 1982 [12, 13] and immediately came into widespread use in many diagnostic clinical assays, in histochemistry, and in diverse research applications. A continuing steady expansion in the use of phycobiliproteins

has occurred since that time [9, 14]. There are many advantages of phycobiliproteins as fluorescent tags over simple organic fluorescent dyes [9].

Isolation of PB5 facilitates studies on characterization of their morphology, composition of phycobiliproteins, and their interaction and reaggregation properties. In the present investigation, a new method has been developed for the rapid isolation of phycobilisomes using a cationic detergent, cetyltrimethylammonium bromide (CTAB). Also, the enhancement of phycocyanin is demonstrated in Spirulina platensis (FTCC 06), a strain which. was found to have the fastest growth rate and highest biomass and protein content among the strains screened. This study aims at increasing the commercial value of this strain which is being cultivated in large scale at Central Food Technological Research Institute, Mysore.

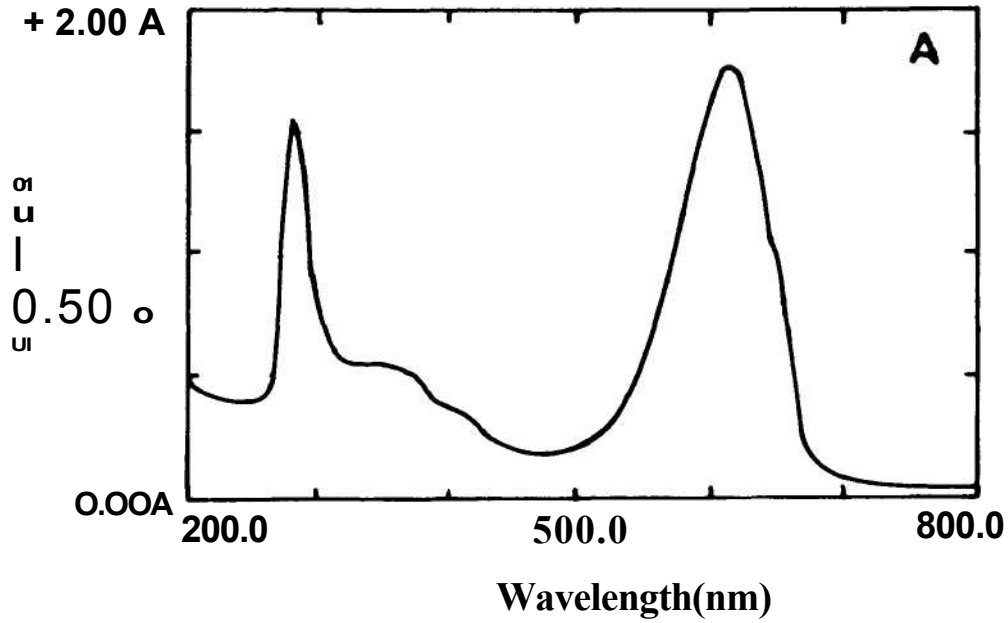
RESULTS AND DISCUSSION

Conditions suitable for cell disruption, solubilization and fractionation of phycobilisomes from Spirulina cells were determined. The release of phycobilisomes from cells treated with detergents was monitored by checking absorption at 615 nm in the 535 fraction **(Fig.** 1A). This released intact phycobilisomes from cells in only 30sec, thereby reducing the chances of proteolysis of the component phycobiliproteins. Chlorophyll contamination in the phycobilisome sample was negligible as evidenced by the minimal absorption at 436 nm, wavelength which indicates the presence of chlorophyll. Phycocyanin represented the major peak with an absorption maxima at 615 nm. Allophycocyanin formed a minor peak as a shoulder at 650 nm. The absorption spectra of the purified phycobilisomes is shown in **Figure 1B.** The spectra of the CTAB-treated 535 fraction and the same fraction further purified by sucrose gradient centrifugation had almost a similar spectral picture. This indicated very low levels of contamination in the preparation.

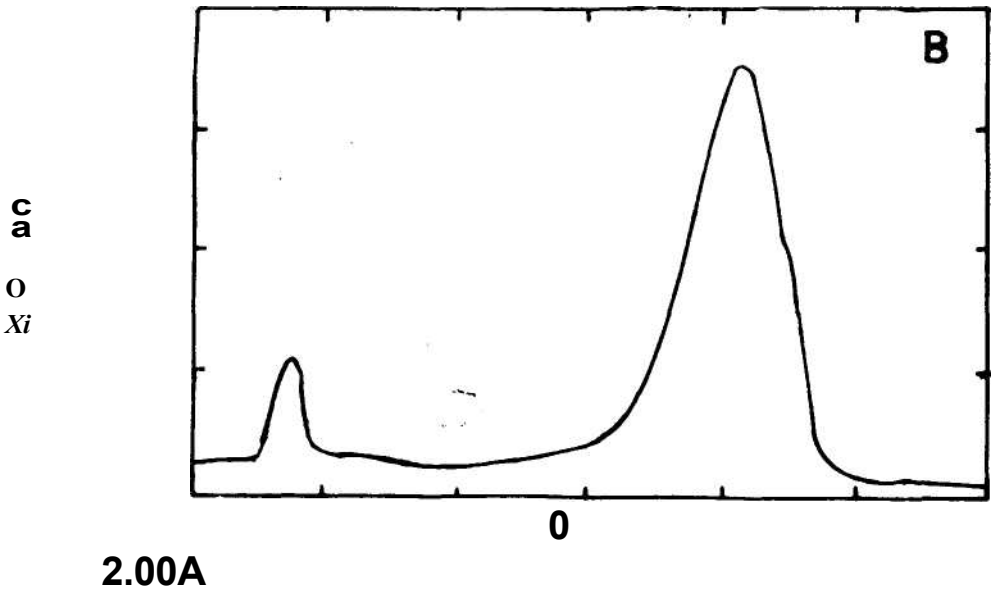
The functional integrity of the phycobilisomes was further examined by fluorescence studies **(Fig.** 2A *&* 28). The fluorescence emission maxima at 20°c

Fig IA. Absorption spectra of CrAB-treated homogenates (535) suspended in 0.75 M K-PO4 buffer

(Absorption at 615 nm is the index for release of phycobilisomes)



1B. Absorption spectra of the purified phycobilisomes

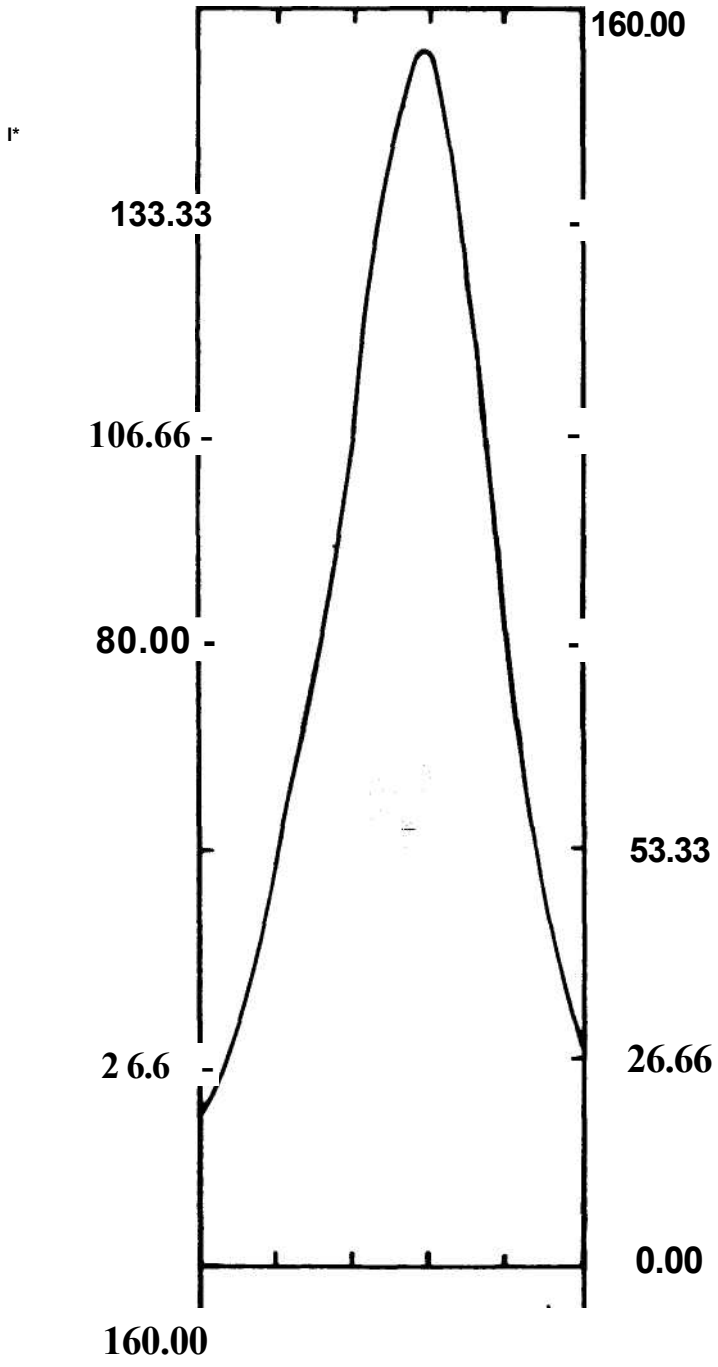


O.OO A 200.0 50 800.0

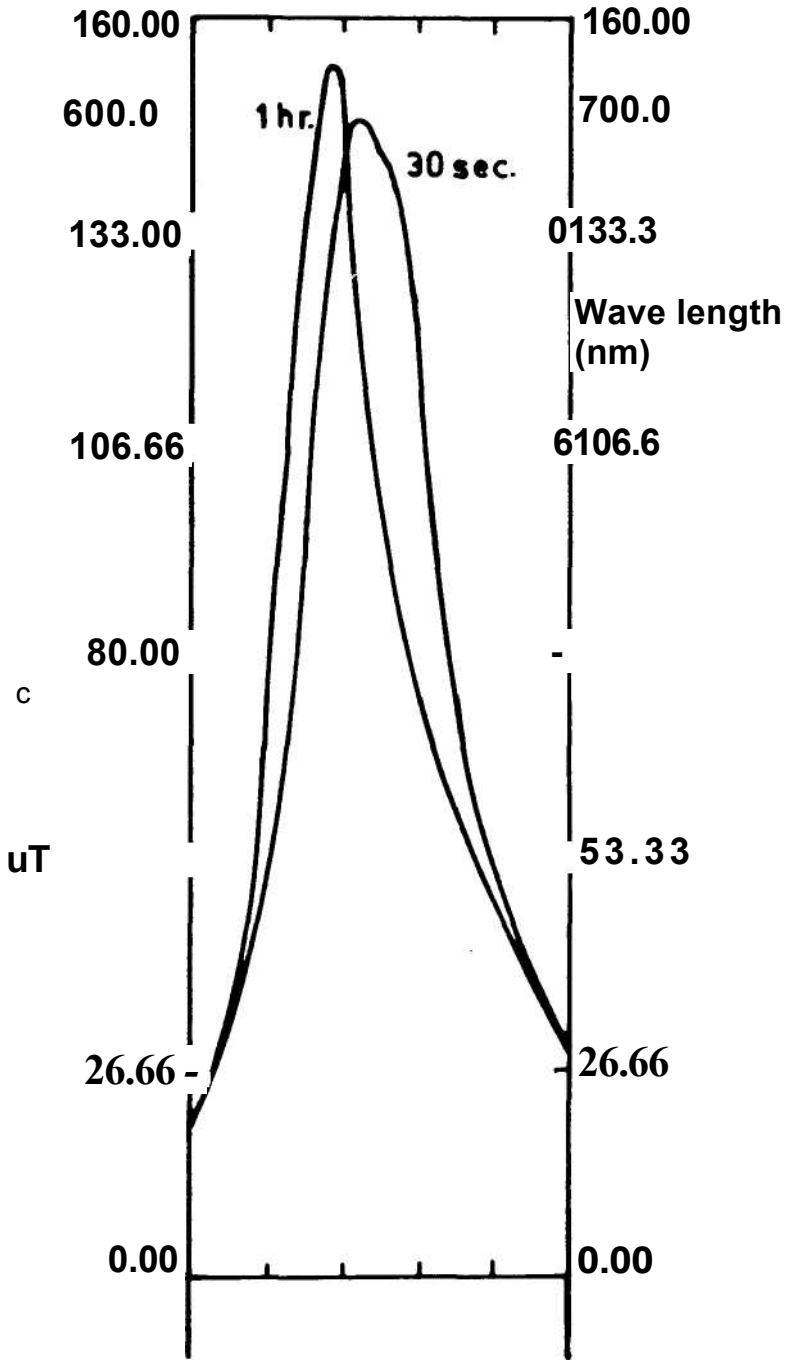
0.0

Wave lengt h(nm)

Figure 2A. Fluorescence spectra of the intact phycobilisomes (Excitation wavelength, 580 nm)



2B. Fluorescence spectra of the dissociating phycobilisomes
Dissociation was carried out by dialyzing the phycobilisome preparation in lower strength buffer (50 mM phosphate buffer)



was observed at 660 nm when excited at 580 nm, indicating that energy absorbed primarily by phycocyanin was effectively transferred to allophycocyanin.

Disruption of the phycobilisomes by decreasing the molarity of the buffer drastically changed the fluorescence emission properties **(Fig.** 2B). However, there was no change in the absorption maxima. The change in fluorescence spectra was followed for an hour. The sequential physical release of phycobiliproteins with concomitant uncoupling is indicated by the shift in fluorescence emission **(Fig.** 2B).

The phycobiliproteins, as reported in the literature, have been resolved by gel electrophoresis in S.platensis and S.maxima [15], and isolated from the former [16]. As per this report [16], both c-phycocyanin and allophycocyanin appear to be oligomeric complexes composed of atleast two different subunits. The α- and β-subunits of c-phycocyanin showed mobilities corresponding to mol. wt. of 20,500 and 23,000, respectively, resulting in an oligomer with a minimum mol. wt. of ca 44,000. Allophycocyanin was found to be composed of subunits with mol. wt. of nearly 18,000 and 20,000 to give an oligomer with a minimum mol. wt. of ca 38,000.

In our study, the SDS-PAGE resolved the two major phycobiliproteins: phycocyanin (2 subunits mol. wt. 18,000 and 19,000) and allophycocyanin (2 subunits mol. wt. 15,000 and 16,500). Four high molecular weight colourless polypeptides of relative mol. wt. 52,000, 47,000, 40,000 and 21,000 and a low molecular weight peptide of mol. wt. 10,000 were observed **(Fig.** 3). Since the biliproteins constitute upto 80% of the phycobilisome, overloading the gel was necessary to identify the linker polypeptides. Overloading prevented resolution of the subunits of phycocyanin and allophycocyanin on the same gel **(Fig.** 3).

PBSs were first purified from the red alga Porphyridium cruentum. They were removed from the photosynthetic membranes with 0.5 M Triton X-I00, fixed with glutaraldehyde, and separated from the rest of the solubilized cellular components by sucrose gradient centrifugation [1]. Later PBS were isolated

Figure 3. 50S-PAGE of phycobilisomes

A) Silver staining

B) Coomassie brilliant blue staining

Lane i - High molecular weight standard mixture containing
1) carbonic anhydrase (29,000)
2) egg albumin (45,000)
3) bovine albumin (66,000)
4) phosphorylase b (97,400)
5) β-galactosidase (116,000)
6) myosin (205,000)

lane ii - Low molecular weight standard mixture containing
1) α-lactalbumin (14,200)
2) trypsin (20,100)
3) trypsinogen (24,000)
4) carbonic anhydrase (29,000)

Lane iii - sample extracted by CTAB treatment (overloaded)

Lane iv - sample extracted by CTAB treatment

without prefixation from the same alga in 0.5 M phosphate buffer on a sucrose gradient at 4°C [16]. The procedure was subsequently modified for some blue-green algae [17, 18], but it was not generally applicable to other species. Further modifications have been made and it was found that the most crucial condition to maintain PBS integrity in addition to high strength is temperature. We have found it is important that isolations be done at about 20-23°C and that the phosphate buffer content be at 0.75 M (pH 7.0). The simplified procedure described here gave high PBS yields.

In all these reports, phycobilisomes have been isolated with unimpaired energy transfer properties using a wide variety of nonionic or zwitterionic detergents (e.g. Triton X-100, Tween 80, Brij 56) in sodium phosphate buffer (pH 7.8 - 8.0) containing high salt concentrations (0.65 - 0.75 M) [17-20]. The procedures required incubation in the presence of detergents for 40 - 60 min at 20°C. Further purification has been achieved by sucrose density gradient centrifugation in a high molarity buffer.

The present study showed that PBS can be isolated rapidly from blue-green algae using CTAB. It is an improvement over the original procedure and its modifications [17, 19, 21-24]. The main criterion used in assessing highly coupled PBS is the fluorescence emission at 660 nm at 20°C. This fluorescence emission was obtained when the entire isolation was carried out at 20°C, including the centrifugation on the sucrose-phosphate step gradient. It has been noted that the sucrose step gradient can be omitted as reported earlier [22], but this depends on the intended purpose of the PBS preparation. The step gradient, and additional subsequent purification steps are required for removal of free phycobiliproteins, soluble cytochromes, adhering proteins, chlorophylls, nucleic acids, and carbohydrate storage products. For example, small amounts of chlorophylls can easily account for the reported phycocyanin (PC) and allophycocyanin (APC) variations between PBS from early and late logarithmic phase cultures [22].

The method described here has distinct advantages over the currently used method of treating with Triton X-100, in that this reduces the

chlorophyll and other contaminations. It also decreases the chances of proteolysis of the component due to the rapid isolation procedure. Also, the structural and functional proper~jes of PBSs are retained as evidenced by spectroscopy and SOS-polyacrylamide gel analysis.

The possibility of the enhancement of the PBS and in turn phycocyanin levels by increasing the nitrate concentration in the growth medium was envisaged since nitrate is an important prerequisite in maintaining the intactness of the core PBS component [25]. Repression of phycobilisome synthesis in the nitrogen deprived cells of Anacystis nidulans was shown by Lau et al. (1977) [26].

Phycocyanin may serve also as a storage material since it has been found that the phycocyanin concentration was highest when S.platensis was cultivated under favourable nitrogen concentrations [27]. If the level of available nitrogen in the medium decreased, or the cultures were completely deprived of nitrogen, a corresponding decrease in the phycocyanin content was observed. No other nitrogen-containing compounds decreased under these conditions, and the decrease of phycocyanin concentration was associated with an increase in the activity of a protease acting on purified c-phycocyanin. If, under these conditions as well as after inhibition of protein synthesis, the cellular concentration of phycocyanins decreased, severe inhibition of photosynthesis and growth was observed [27]. Dual role of c-phycocyanin, as a storage material and an accessory pigment in photosynthesis, was inferred also from the fact that, unlike other reserve substances that accumulate at the end of the growth phase or in the final stage of the life cycle, c-phycocyanin was synthesized mainly during the logarithmic phase of growth [28].

In our experiments conducted for the enhancement of phycocyanin levels, there was nearly 8, 18, 27% increase over the control when the nitrate concentration was raised to 2, 3 and 4 times respectively **(Table 1).** Above these levels, the growth was found to be impaired (decreased to 81% of the original growth). Unlike in other cyanobacteria [29-34], there was no increase in the synthesis of PBS at low light intensity in Spirulina, indicating the absence of chromatic adaptation in the organism.

Table 1. Effect of nitrate on protein, chlorophyll a anc phycocyanin content of Spirulina platensis (FTCC 06)

Nitrate conc. (g/L)	Protein (mg/g DW)	Chlorophyll a (mg/g DW)	Phycocyan i n (mq/g DW)
2.5*	638.4 (22.8)	11.76 (0.50)	**173.1** (5.5)
5.0	641.2 (27.2)	11.90 (0.48)	186.5 (5.5)
7.5	648.7 (29.8)	12.34 (0.50)	204.6 (8.7)
10.0	652.5 (29.9)	14.51 (0.51)	219.8 (9.9)

* Control
Results presented are averages of three independent experiment: carried out in triplicates (±SD)

A few potential microalgal products like phycocyanin are unique to blue-green algae, while others can be obtained from various sources β-carotene, polysaccharides, etc.). The public concern had led to an increase in the usage of natural food colours [35], and phycocyanin is a rare bright blue pigment among the natural food colours. Phycocyanin from Spirulina has been used as a superior source of natural colorant [36]. This pigment has also found wide application in the cosmetic industry in Japan. Its potential as a base in ointment, creams, lotions, etc. has been emphasized [37]. Additionally, phycocyanin has been indicated to be a highly safe pigment by various studies on Spirulina [38]. It can be presumed that phycocyanin can be more extensively utilized in future. An enzyme immunoassay by using mouse monoclonal antibodies for the screening and semi-determination of phycocyanin in foods is reported [39].

As indicated earlier, phycocyanin is also a novel fluorescent tag [12, 13] and has widespread use in many diagnostic clinical assays. In addition, phycocyanin is shown to have antioxidant properties. In a Japanese study, rats with liver cancer showed a higher rate of survival in the Spirulina-phycocyanin treated group [40].

The annual market for phycocyanin is US $ 5-10 million. The value of phycocyanin used in diagnostics is >$ 10,000/kg and that used as food color is >$ 100/kg [41]. Spirulina is the most widely reported source of phycocyanin. The enhancement achieved in phycocyanin levels of Spirulina by this study would be economically viable since the objective of microalgae biotechnology is to produce specific products which have more substantial commercial value and importance.

EXPERIMENTAL

Chemicals used

Cetyltrimethylammonium bromide, acrylamide, bis-acrylamide, Tris and molecular weight markers were obtained from Sigma Chemicals and Co., USA.

Other chemicals used were of the highest purity available locally. Deionized and double distilled water was used throughout.

Preparation and characterization of phycobilisomes

Spirulina platensis (FTCC 06) was grown in Zarrouk's liquid medium [42] at 28+1°C with constant shaking (90 rpm), at a light intensity of 100 μE m-2 s-1 in 1L shake flasks. Mid logarithmic phase cells in medium (O.D-1.0 at 560 nm) were incubated with 0.01% CTAB for 30 sec at 20°C and immediately harvested through two layers of cheese cloth, washed and resuspended in 0.75 M potassium phosphate buffer (pH *7.0)* at a ratio of 1:10 (wet wt. by volume). The cell suspension was sonicated for 1 min at 12 K/cycle/sec in an ice bath. The sonicate was centrifuged at 35,000 g for 20 min at 20°C. The resulting supernatant was layered onto a sucrose step gradient consisting of 2.0, 1.0, 0.5 and 0.25 M sucrose solutions, 1 ml each, prepared in 0.75 M K-PO4 buffer (pH 7.0). Centrifugation was done in a preparative ultracentrifuge (Beckman L5-50B) using a SW 28 rotor at 60,000 rpm for 4 hrs at 20°C. The phycobilisomes were recovered as a deep blue band and used for spectroscopic measurements and SDS-PAGE.

The recovered phycobilisomes were dialyzed against 50 mM potassium phosphate buffer (pH 7.0). The extent of dissociation was checked after half an hour and one hour by measuring the spectral properties of the dissociated phycobilisomes.

Absorption spectra were recorded at room temperature on a Shimadzu UV spectrophotometer (UV-160A). Fluorescence emission spectra were measured with a Shimadzu spectrof luorophotometer (RF 5000). The excitation wavelength used was 580 nm.

SDS-PAGE was done on a 12.5% SDS-acrylamide gel in a Tris-glycine buffer [43]. The mini slab gel electrophoretic apparatus (15x 18x 15, vertical; Bangalore Genei Pvt. Ltd.) was used with a power supply of 50 volts. Protein bands were detected by both silver staining [44] and Coomassie blue

staining. High molecular weight standard mixture containing carbonic anhydrase (29,000), egg albumin (45,000), bovine albumin (66,000), phosphorylase b (97,400), p-galactosidase (116,000), myosin (205,000) and low molecular weight standard mixture containing oc-Iactalbumin (14,200), trypsin (20,100), trypsinogen (24,000) and carbonic anhydrase (29,000) were used as markers.

Enhancement of phycocyanin

Cultures of Spirulina platensis were grown in Zarrouk's liquid medium at 32°C with constant shaking (90 rpm), at a light intensity of 100-150 μ*E* m-2 s-1 in 1L shake flasks. Cells pregrown at these normal growth conditions with 2.5 g NaN03/L were centrifuged (1,00Oxg, 10 min) and harvested at an actively growing stage (0.8 0.0. at 560 nm). The collected cells were resuspended in stress medium containing higher concentrations (5.0, *7.5* and 10.0 g/L) of nitrate **(Table 1).** Phycocyanin content was estimated [45] after 10 days.

Estimation of phycocyanin

The cells were harvested from a known volume of the culture and pelleted by centrifugation (1,000xg, 10 min). Washed the pellet with distilled water and resuspended the pellet in 6 ml of 0.01 M sodium phosphate, pH 7.0:0.15 M sodium chloride (buffered saline). Immersed the tube containing cell suspension in an ice bath and sonicated the cells using an ultrasonicator for 1 min at 12 K/cycle/sec. Centrifuged the sonicated cells to remove membrane fragments (5,000xg, 30 min) and collected the supernatant which contained phycobiliproteins and other soluble proteins. Read the absorbance at 615 and 652 nm and calculated the concentration of C-phycocyanin using the equation derived from extinction co-efficient of purified phycobiliprotein [5].

$$\text{C-phycocyanin} = \frac{\text{A615} - 0.474\,(\text{A652})}{5.34}$$

The concentration of phycocyanin in a given volume of culture was determined as follows.

$$\text{Phycocyanin (mg/ml culture)} = \frac{C \times Ve}{Vc}$$

where, C is the concentration of phycocyanin
Ve is the volume of the extract
Vc is the volume of the culture

Phycocyanin content was expressed in terms of algal dry weight using the standard graph of optical density at 560 nm of the culture vs. dry weight.

Protein [46] and chlorophyll [AOAC Official Methods of Analysis, 1984] were also estimated.

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CHAPTER 4

SUPEROXIDE DISMUTASE OF *SPIRULINA*

SUMMARY

Superoxide dismutase (EC 1.15.1.1), was purified from Spirulina maxima (FTCC 03) followed by enzymological characterization and evaluation of its antioxidant properties. The various stages of purification involved were ammonium sulfate fractionation, ion-exchange chromatography and molecular sieving. The final enzyme preparation showed nearly 79 fold purification with a recovery of 69%. The native as well as 50S-molecular weight was found to be 22,000. The enzyme retained 50% of the activity when exposed to 50°C for 30 min, whereas, only 6% of the activity remained at 60°C. The form of SOD was also identified as iron containing protein.

The purified enzyme preparation exhibited an antioxidative effect of 0.94 in oxidation studies with linoleic acid emulsion. The addition of bovine-catalase reduced the antioxidative effect of purified SOD. Attempts were made to immobilize the enzyme on matrices like gelatin and DEAE Sephadex A-50 and their specific activity compared with that of the free enzyme.

The catalases and superoxide dismutases (SODs) are the most efficient antioxidant enzymes. Their combined action converts the potentially hazardous superoxide radical (.02-) and hydrogen peroxide (H202) to water (H2O) and molecular oxygen (02), thus averting cellular damage [1].

SOD (EC 1.15.1.1) was first isolated from bovine blood as a green copper protein [2] whose biological function was believed to be copper storage. Over the years, the er.zyme has been variably referred to as erythrocuprein, indophenol oxidase and tetrazolium oxidase. The enzyme is ubiquitous, being widely distributed among O_2-consuming organisms, aerotolerant anaerobes, and some obligate anaerobes [3]. SODs catalyze a disproportionate reaction at a rate very near that of diffusion [4]. To accomplish this reaction, the mechanism employs an alternate oxidation/reduction of the respective metal associated with the enzyme. Three distinct types of SODs, based on the metal ion in their active sites, have been observed from a wide range of organisms examined. Thus, there are SODs that contain copper and zinc (Cu/ZnSOD), manganese (MnSOD), or iron (FeSOD) [5]. With a few exceptions, Cu/ZnSODs are generally found in the cytosol of eukaryotic cells and chloroplasts; the MnSODs are found in the matrix of mitochondria and in prokaryotes; the FeSODs

are generally found in prokaryotes and have been reported to exist in some plants [5].

The blue-green alga, Plectonema boryanum, contains both Fe- and MnSOD. The localization of MnSOD in the thylakoids and that of the FeSOD in the cytosol of three species of blue-green algae: f.boryanum, Anabaena variabilis and Anacystis nidulans has also been shown [6]. Although there is a report indicating the presence of FeSOD in Spirulina [7], SOD has not been purified and characterized from this alga. In view of its applicability as a potential antioxidant in lipid foods and its value addition to Spirulina (a widely used health food supplement [8]), SOD was thought to be the most suitable enzyme for the present study.

SOD was purified from Spirulina maxima followed by enzymological characterization. Further, the antioxidative effect of SOD purified from Spirulina in a model system of linoleic acid is assessed. Attempts were also made to immobilize the purified enzyme preparation using DEAE-Sephadex A-50 and gelatin. The specific activity of the immobilized enzyme was compared with that of the free enzyme and the advantages and disadvantages of each of the above matrices discussed.

RESULTS AND DISCUSSION

Enzyme Purification and **Characterization**

A summary of the results for the purification of SOD from Spirulina is presented in **Table 1.** The starting material, i.e., pelleted Spirulina cells from fresh cultures, amounted to about 0.4 g dry weight. This yielded nearly 8,300 units of SOD activity/lOg OW.

The crude extract was subjected to 55-85% ammonium sulphate fractionation which was determined after standardizing the optimal range for fractionation. The enzyme got bound to the ion-exchanger DEAE Sephade~ A-50 resulting in the removal of the contaminating proteins when eluted with low strength buffers. The enzyme was eluted finally with 0.05 M phosphate buffer, pH 7.8. **Figure 1** gives the elution profile of SOD on the ion-exchange column. The fraction from the gel-filtration column resulted in an enzyme preparation

which was 79-fold pure with a recovery of 69% **(Table 1).** The elution pattern on the gel filtration column in given **Figure 2.**

The electrophoretic patterns of the native and SDS-PAGE are depicted in **Figures 3 A and D** in which the band of interest, i.e. SOD is indicated by an arrow. The crude enzyme extract of Spirulina maxima showed only one distinct band of SOD in PAGE, when subjected to activity staining (Fig. 38). The purified enzyme preparation was found to be electrophoretically homogenous on native gel which is clearly shown by silver staining **(Fig.** 3A) whereas, on SDS-PAGE **(Fig.** 3D), a few minor bands were visualized which indicated that there were minor contaminants, which could be detected by silver staining which is sensitive upto 0.2 ng level of proteins [9].

Table 1 : Purification profile of SOD from Spirulina maxima

Stages of purification	Total protein (mg)	Total activity (Units)	Specific (U/mg protein)	Fold purification	(%)
Crude enzyme extract	246.13	331.67	1.35	0	100
55-85% $(NH_4)2SO_4$ fraction	240.50	329.79	1.37		99.4
Ion-exchange fraction	12.13	296.73	24.46	18	89.5
Gel-filtration fraction	2.17	230.12 106.05		79	69.4

The results are averages of three independent experiments.

Figure 1. The purification profile of SOD from Spirulina on ion-exchange column (DEAE Sephadex A-50)

The column was equilibrated and eluted with 0.015 M phosphate buffer, pH 7.8. The elution was carried out with increasing strengths of the buffer (0.0 1 5, 0.02, 0.03, 0.04 and 0.05). The enzyme got eluted in 0.05 M buffer. Volume of each fraction was 5.0 mI.

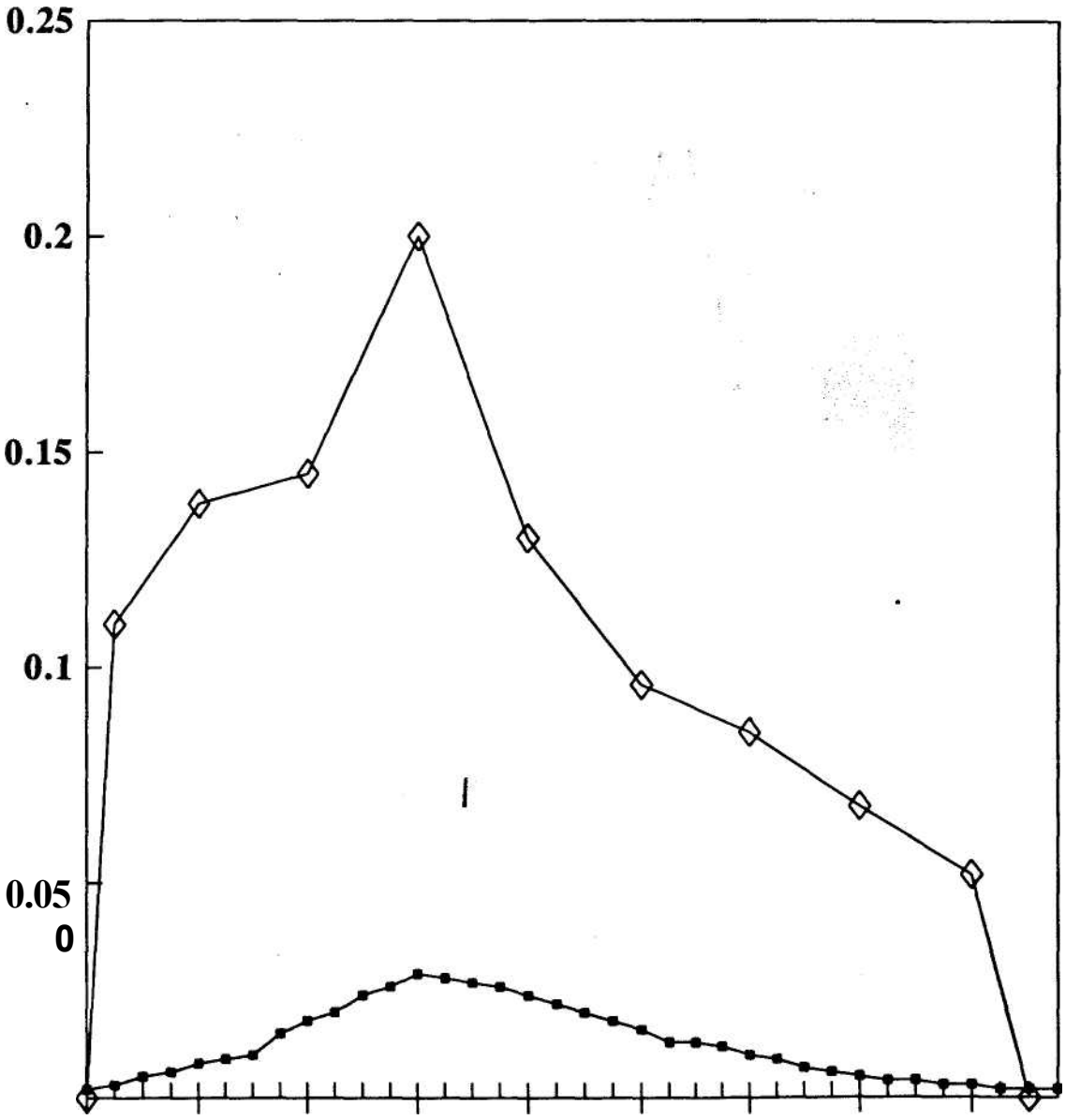


Figure 2. The purification\ profile of SOD from Spirulina on gel-filtration column (Sephadex G 50-150)

The flow rate was 0.32 ml/min. The void volume was 35.0 mI. Volume of each fraction was 1.5 mI.

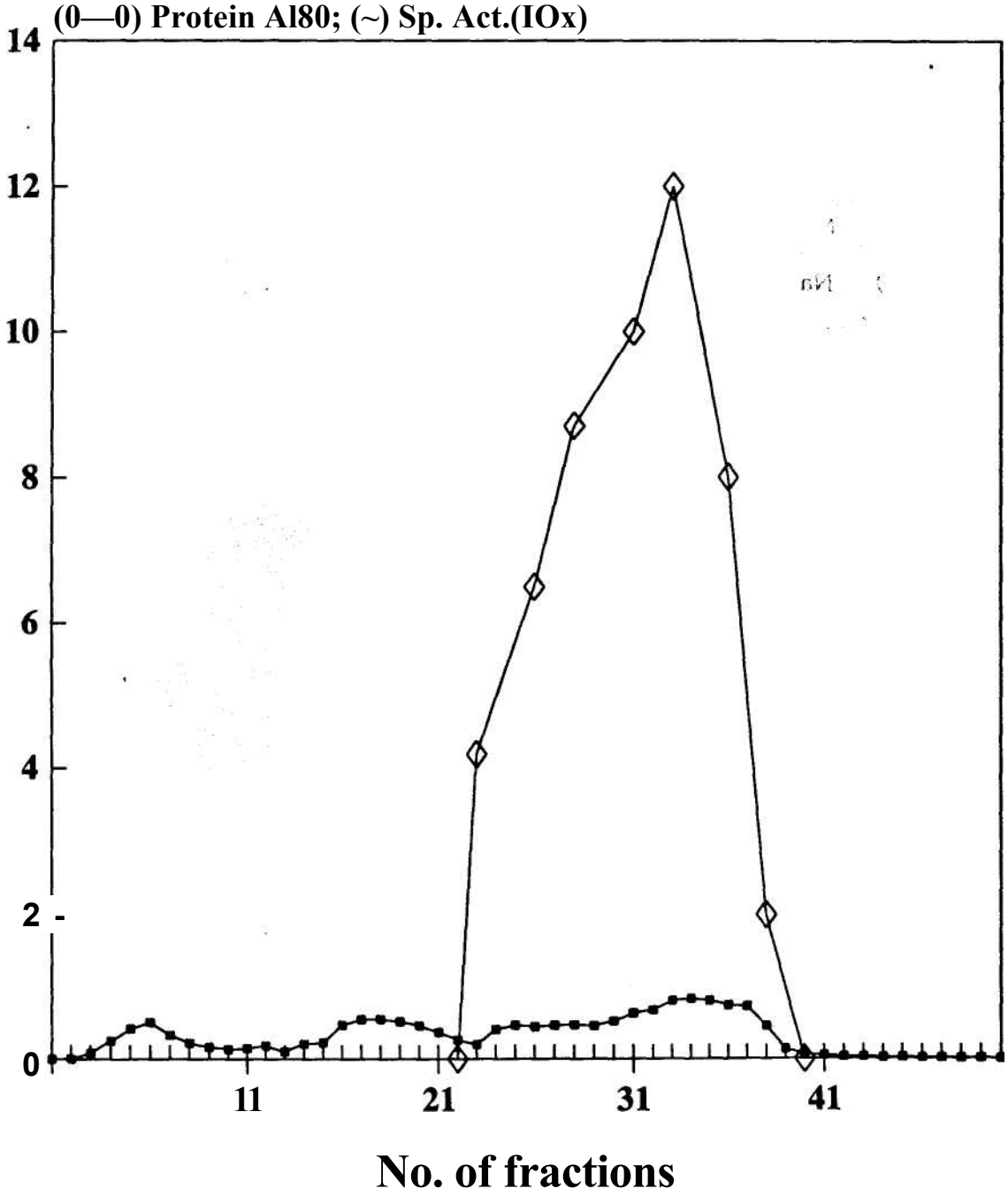


Figure 3. The electrophoretic patterns of Native and 5DS-PAGE of SOD from Spirulina maxima

A) Native-PAGE (Silver staining)

B) Native-PAGE (Activity staining)

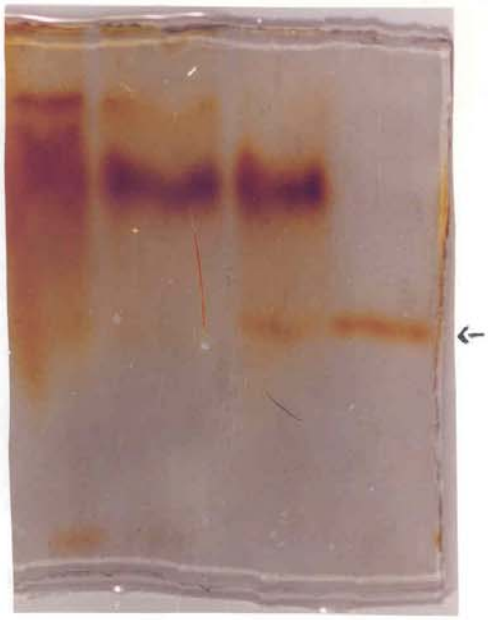
C) Native-PAGE (Silver and activity staining)

i) Crude enzyme fraction ii) 55-85% Ammonium sulfate fraction iii) lon-exchange fraction iv) Gel-filtration fraction
(Common to A, B and C)

D) SDS..PAGE (Silver staining)

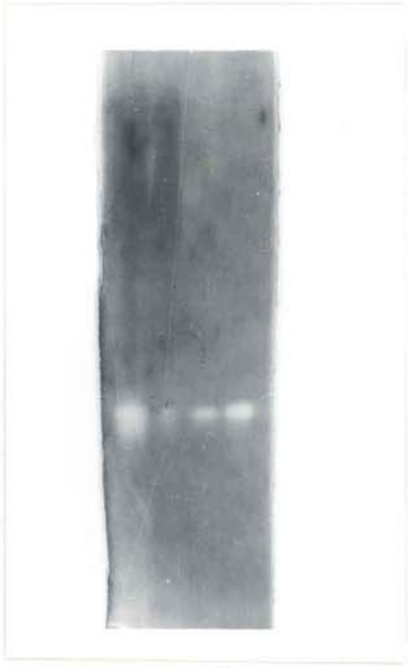
i) Mol. wt. markers :
1) carbonic anhydrase (29,000)
2) trypsinogen (24,000)
3) β-lactoglobulin (18,400)
4) lysozyme (14,300)
ii) Purified SOD

A



i ii iii iv

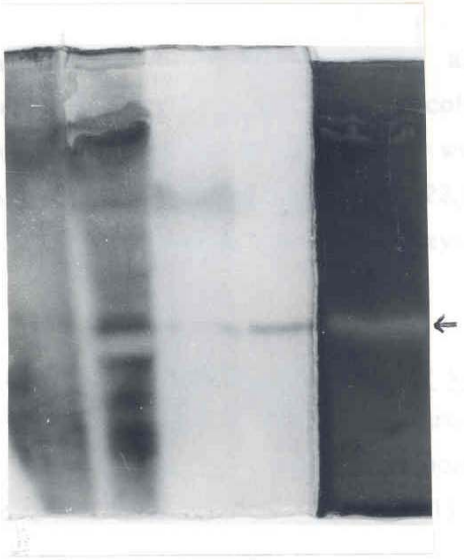
B



i ii iii iv

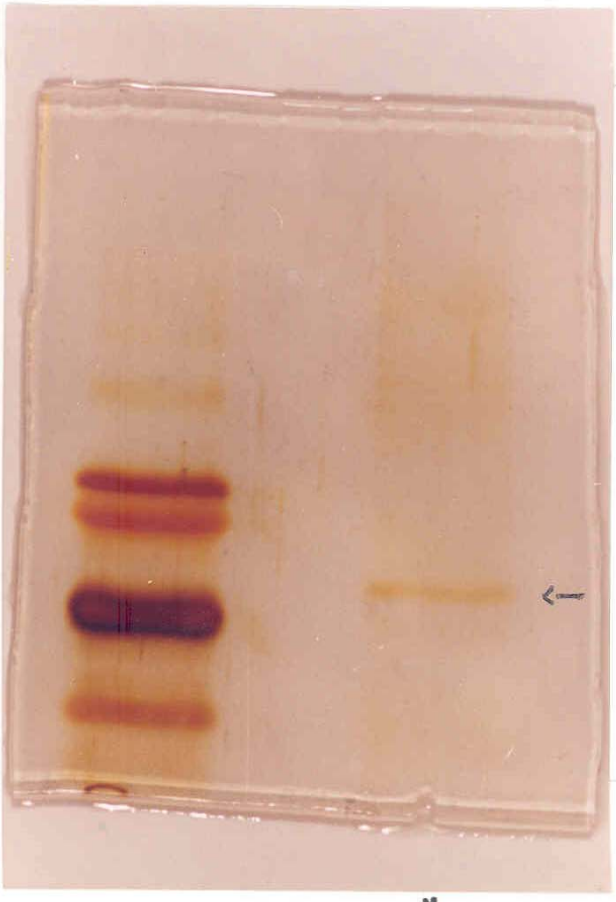
i ii iii iv

C

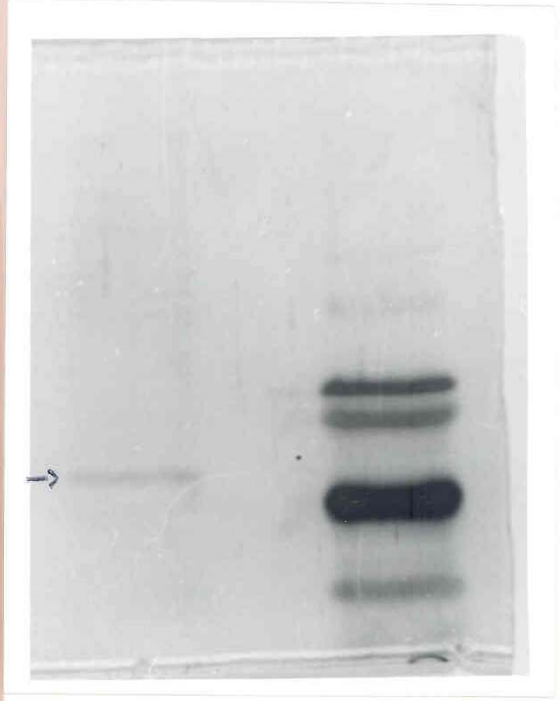


i ii iii iv i

D



i ii



4
3
2
1

ii i

A single peak of activity was obtained after gel permeation chromatography of purified material on a calibrated column of Sephadex G 50-150. The elution volume corresponded to a molecular weight of 22,000 Da **(Fig.** 4). A single subunit band of molecular weight around 22,000 Da was detected on SDS-PAGE **(Fig.** 3D). The data suggest that the enzyme could probably be a monomer.

SOD purified from nitrogen fixing Azotobacter vinelandii is reported to be composed of two identical subunits with an apparent molecular weight of 21,000 Da [10]. The enzyme from soya beans has a molecular weight of 36,300 Da and a subunit molecular weight of 18,000 Da [11] and that from brussels sprouts (Brassica oleracea L.) is composed of two subunits of molecular weight 16,000 and 19,000 Da, respectively [12]. Most of the reports on purification of SOD from various sources other than algae indicate that the enzyme exists as a dimer or multimer and it seems to be present in the monomeric form in this alga.

The properties of the purified enzyme preparation are listed in **Table 2.** The enzyme could retain 50% of the activity when exposed to 50°C for half an hour. At 60°C for 30 min., the enzyme had only 6% of the original activity **(Fig.** 5). The temperature optima could not be determined because of the influence of the temperature on the reduction of NBT. The optimum temperature for the reduction of NBT is 25°C and this reaction was inhibited at very low «25) or high temperatures (>25) which made the SOD assay difficult. It could be assumed from the observations, that SOD had an optimum temperature of 10-20°. The temperature optimum is not reported widely for SOD from other sources due to this reason. The enzyme had a broad pH optima with the activity unchanged over the range from 6-9. Below pH 6, it was difficult to assess the activity because the reduction of NBT fell off very rapidly below pH 6.

In inhibition studies, there was complete inhibition of the enzyme activity by hydrogen peroxide at a level of 20 }LM. Since the lack of inhibition by hydrogen peroxide is considered to be specific for Mn-containing

Figure 4. Molecular weight determination of the native protein

Standards used: egg albumin (45,000); carbonic anhydrase (29,000); trypsinogen (24,000); 3-lactoglobulin (18,400) and lysozyme (14,300)

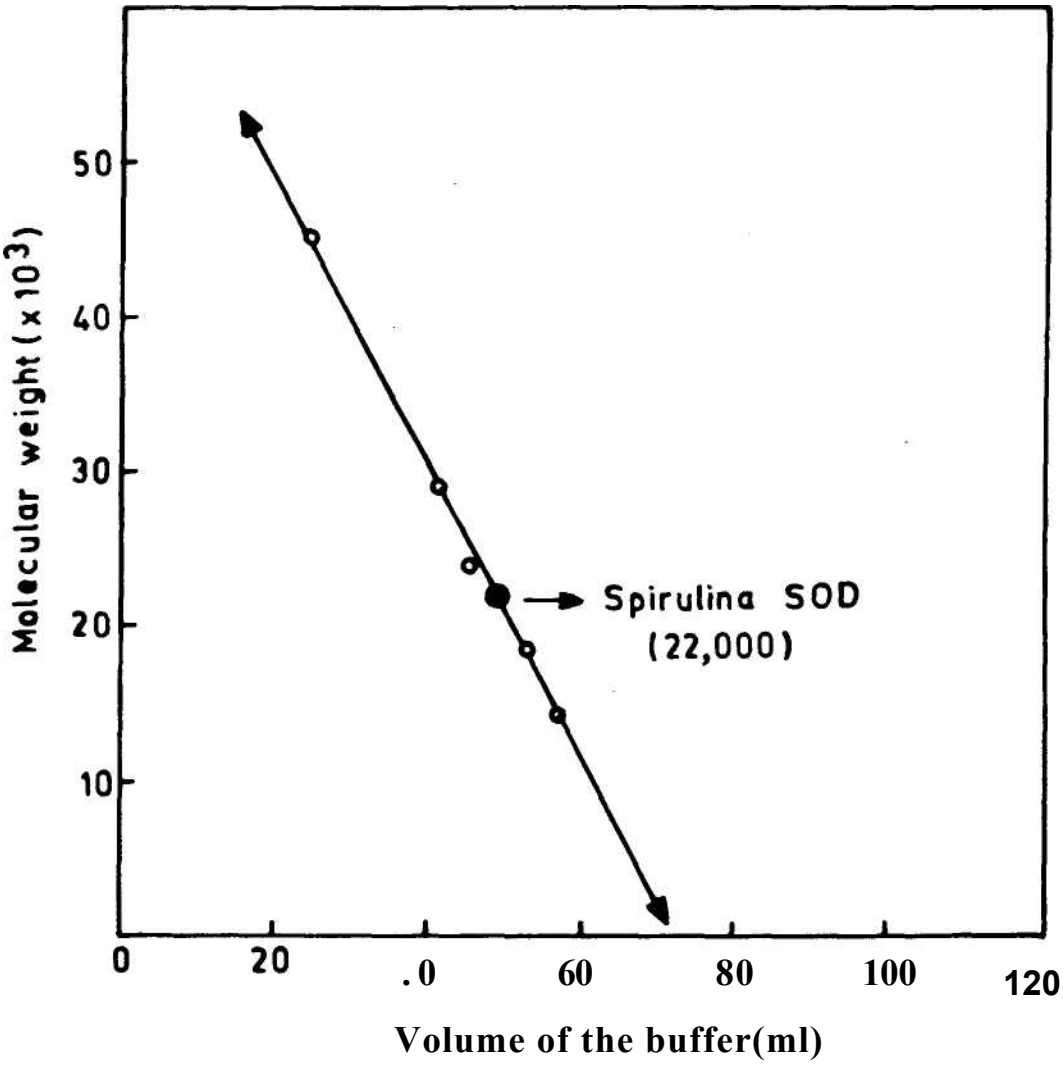
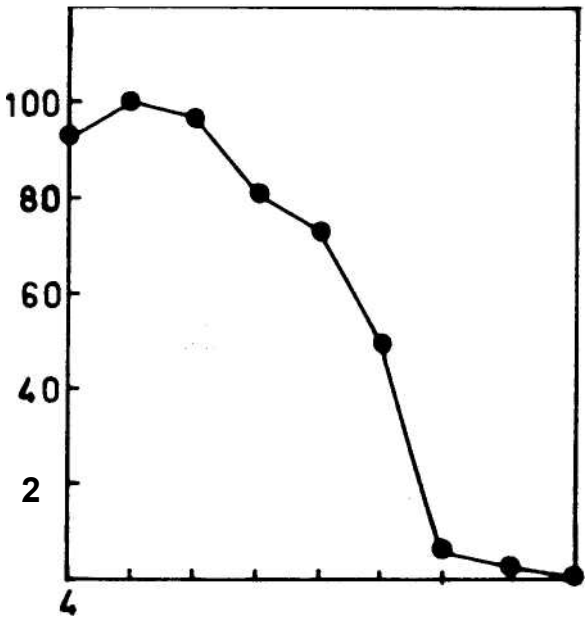


Table 2 : Properties of purified SOD

Temperature stability	50°C (50% activity)
pH optima	pH 6 - 9
Native Molecular weight	22,000
SOS Molecular weight	22,000
Inhibition studies	
Hydrogen peroxide (20 µ*M)*	100% inhibition
Sodium cyanide (1 mM)	No inhibition
Sodium azide 12.5 µM)	28% inhibition
(25 µM)	60% inhibition
(50 µ*M)*	100% inhibition

Figure 5. Temperature stability of SOD



SOD and not for the other metal forms [6, 13], this result probably indicated the absence of MnSOD. Analysis of the activity inhibition by either azide ,or cyanide [14] suggested that this SOD could mostly be a Fe-containing enzyme. The inclusion of KCN (1 mM) in the NBT-assay did not result in the inhibition of the purified SOD, indicating that the activity was not due to Cu/Zn-SOD. Only MnSOD and FeSOD are unaffected by this concentration of cyanide [14]. Sodium azide caused 28% inhibition at 12.5 μM, 60% inhibition at 25 μM and complete inhibition at 50 pM. With all the above observations it was assumed that the enzyme is Fe-containing SOD. The presence of iron in the purified SOD from Spirulina was confirmed by the detection and quantification of iron by atomic absorption spectrophotometry. An iron content of approx. 80μg/mg of the protein was read. Manganese, zinc and copper were not detected.

There is a general agreement that Fe-SOD is a constitutive enzyme in prokaryotes [15]. Lumsdon and Hall (1974, 1975) [7, 16] reported cyanide-insensitive SOD activity in Spirulina platensis. This indicated the presence of Fe, as in some of the bacterial and cyanobacterial dismutases, rather than Cu/Zn-SOD from chloroplasts. MnSOD is also present in some blue-green algae in addition to Fe-SOD, for eg., Plectonema boryanum, Anabaena variabilis and Anacystis nidulans [6]. Generally, prokaryotes, protozoa and most algae lack Cu/Zn-enzyme, but contain Fe- and/or Mn-superoxide dismutase [17, 18]. **Table 3** gives a comparison of the SODs from various sources.

Lumsden et al. [16] made an electrophoretic survey of the occurrence of SOD in photosynthetic organisms, both prokaryotic (bacteria and blue-green algae) and eukaryotic (red and green algae) and have confirmed the presence of cyanide-insensitive SOD activity in all the organisms.

Antioxidant Properties of the Purified SOD

Oxidation studies with linoleic acid emulsion showed that the crude SOD preparation from Spirulina was only moderately antioxidative, as compared to commercial bovine SOD **(Table** 4). This shows that the crude enzyme extract contained prooxidative or inhibitory components and hence purification to some extent was needed prior to further studies on the antioxidative effect.

Table 3 : Comparison of SOD from Spirulina with SOD from otheJ sources

Source	Form	Mol. wt.	Subunit mol. wt.	Ref.
E.coli	Mn-SOD	39,500	2 identical	Keele et ~l. (1970)
Tritricho-		38,000	subunits	Lindmark & Muller
monas foetus				(1974)
Monocerco-		38,000		Lindmark & Muller
tnonas sp.				(1974)
Pleurotus	Mn-SOD	80,000	_	Lavelle et al.
olearius				(1974)
Plectonema	Fe-SOD	41,700	2 identical	Asada et al. (1975)
boryanum	&	22,000	subunits	Present study
Spirulina	Mn-SOD		single	
maxima	Fe-SOD		subunit	

Keele et al. (1970). J. Bioi. Chern. **245:** 6176-6181.
Lindmark ~ Muller (1974). J. Biol. Chem. **249:** 4634-4637.
Lavelle et al. (1974). Biochemie. 56: 451-458. Asada et -al~
(1975). J. Bioi. Chern. **250:** 2801-2807.

Table 4 : Antioxidative effect (A.E.) of SOD from Spirulina on the oxidation of linoleic acid

SOD activity	Spirulina SOD (A.E.)			BovinE
Unit/ml emlllsion	crude preparation	55-85% $(NH_4)_2S0_4$ fraction	Final purified fraction	SOD (A.E.)
1	-0.03 (0.01)	0.41 (0.03)	0.45 (0.03)	0.46 (0.04)
5	0.41 (0.04)	0.68 (0.05)	0.75 (0.06)	0.83 (0.05)
25	0.20 (0.03)	0.61 (0.04)	0.94 (0.05)	0.97 (0.05)

The results are averages of three independent experiments. The standard deviation is given within brackets.

A.E. is expressed in values ranging from 0 to 1, where 0 represents no antioxidative effect while 1 is complete inhibition of the oxidation (Negative values indicate a prooxidative effect).

Ref. Lingnert et al. (1979). J. Food Process. & Preser. 3: 87-103. --

Table *4* gives a comparison of the antioxidative effect of different fractions of SOD from various stages of purification viz., the crude Spirulina extract, fraction from ammonium sulfate precipitation (55-85%), the final fraction after passing through ion-exchange and gel-filtration columns. This in turn, is compared to commercial bovine SOD at three different levels of enzyme activity. The bovine enzyme showed a strong antioxidative effect, which increased with increasing concentration. As mentioned above, the crude enzyme preparation of Spirulina was considerably less antioxidative or, at the lowest concentration even somewhat prooxidative. The antioxidative effect also decreased when the concentration of the crude enzyme was increased from 5 to 25 units/mI. The ammonium sulfate precipitated fraction showed an antioxidative effect comparable to that of the bovine SOD at the lowest concentration. However, the increase of the SOD concentration from 5 to 25 units/ml led to a decrease of the antioxidative effect as above. This suggests that the precipitated fraction still contained prooxidative components. The finally purified fraction exhibited increasing antioxidative effect with increasing concentration which is similar to that of bovine SOD.

Since SOD dismutates superoxide radicals into oxygen and hydrogen peroxide, an effective protective system would preferably also degrade the hydrogen peroxide. In theory, the combination of SOD/catalase would therefore be a good antioxidative system. For this reason, the influence of catalase on linoleic acid oxidation was investigated **(Table .5).** The table shows that the addition of bovine catalase reduced the antioxidative effect of purified SOD from Spirulina. The catalase had a prooxidative effect in the linoleic acid system in agreement with the earlier reports [19, 20].

Lipid peroxidation is a major cause of quality deterioration in foods and is a significant factor affecting the stability and nutritional value of many foods. It occurs by a free-radical chain reaction [21]. It can be initiated either by enzymes such as lipoxygenases, which catalyze the formation of hydroperoxides, by metal ions and other constituents or possibly by irradiation, which gives rise to free radicals [22].

Table 5 : Antioxidative effect (A.E.) of purified SOD froB Spirulina alone and in combination with bovine catalase on the oxidation of linoleic acid

SOD activity (Unit/ml emulsion)	A.E.	
	Spirulina SOD	Spirulina SOD+bovine catalase (0.4 U catalase per U of SOD)
1	0.46 (0.04)	0.35 (0.03)
5	0.74 (0.05)	0.64 (0.03)
25	0.96 (0.05)	0.92 (0.05)

The results are averages of three independent experiments and the standard deviation is given within brackets

In order to prevent such autoxidation, various oxidation inhibitors may be added. Inhibitors of autoxidation may be classified into groups according to their mechanism of action. **Table 6a** gives the classification of lipid oxidation.

The most readily acceptable oxidation inhibitors are common food ingredients, as their use is not limited by legislation. Many foods contain compounds that possess antioxidant activity **(Table 6b),** but some foods are of limited use as additives as they impart a specific flavour, aroma or colour to the finished product; furthermore, those that have low antioxidant activity or low solubility in lipids are of limited use in the stabilization of edible oils and fats, although they may be used in fatty foods. Purified 'nature-identical' substances or extracts obtained from foods that have antioxidant activity may be used as well [23].

Extracts from algae such as Chlorella spp. and Scenedesmus acutus contain various antioxidant compounds. Fujimoto ~!, 1: [24] isolated natural brominated hydroxylated benzaldehydes and benzylalcohols from red algae; the compounds had antioxidant activities comparable to those of synthetic phenolic antioxidants.

The use of SOD as an antioxidant in food was first discussed by Michelson and Monad (1975) [25], who showed that SOD inhibited peroxidation in browning fruits and vegetables and that a number of model oxidation reactions were also inhibited. Other workers have shown the antioxidant effect of SOD in milk [26, 27] and in fatty acid model systems containing xanthine oxidase [28] and lipoxygenase [29]. Lingnert et al: (1989) [20] have confirmed the inhibitory effect of SOD from yeast (Saccharomyces cerevisiae) on the oxidation of ascorbic acid, autoxidation of linoleic acid and Cu2+ -catalyzed oxidation of cholesterol. **Table 7** gives an account of the applicability of SOD as an antioxidant in foods.

Enzyme Immobilization

When the enzyme was immobilized on DEAE Sephadex A-50, the blue colour formed after the enzyme assay got bound to the column material which was

Table 6a : Classification of lipid oxidation

Type of inhibitor	Mechanism of action
Antioxidants	Reaction with free radicals,
Synergists	Increasing antioxidant activity
Retarders	Reducing hydroperoxides without forming free radicals
Metal scavengers	Inhibiting the ability of heavy metals to catalyze the production of free radicals
Singlet-oxygen quenchers	Deactivating singlet oxygen, whicl may initiate the free~radica: chain reaction

Table 6b : Sources of the main natural oxidation inhibitors

Sources	Oxidation inhibitors
Oils and oilseeds	Tocopherols and tocotrienols; sesamol and related substances olive oil resins; phospholipids
Oat and rice brans	Various lignin-derived compounds
Fruits and vegetables	Ascorbic acid; hydroxycarboxylic acids; flavonoids; carotenoids
Spices, herbs, tea, cocoa	Phenolic compounds
Protein and protein hydrolysates	Amino acids; dihydropyridines; maillard reaction products

Table 7 : An account of the applicability of SOD from various sources as an antioxidant in foods

Use	Reference
Inhibition of peroxidation in browning fruits and vegetables	Michelson & Monad (1975)
Antioxidant effect in milk	Aurand et al. (1977) Hill et al. (1977)
Antioxidant effect in fatty acid model systems containing i) xanthineoxidase i) xanthineoxidase	Kellogg & Fridovich (1975) Richter et al. (1975)
Inhibitory effect of SOD from yeast (Saccharomyces cerevisiae) on i) the oxidation of ascorbic oxid ii) autoxidation of linoleic acid iii) Cu^{2+}-catalyzed oxidation of cholesterol	Lingnert et al. (1989)

Michelson & Monad (1975). US Patent 3910521
Aurund aL. (1977). J. Dairy Sci. 60: 363-369.
Hill et al. (1977). NZ J. Dairy Sci. Technol. 12: 69-77.
Kellogg & Fridovich (1975). J. Biol. Chem. **250:** 8812-8817
Richter et al. (1975). FEBS Lett. 51: 300-303.
Lingnert et al. (1989). J. Agric. Food Chem. 37: 23-28.

removed by phosphate buffer of higher strength (0.03 M). The specific activity of the immobilized enzyme was found to be 88% of the activity shown by the free enzyme. The assay could be repeated upto 5 times without any appreciable loss in activity, after which there was a gradual reduction in activity (10-15% per usage) **(Fig.** 6).

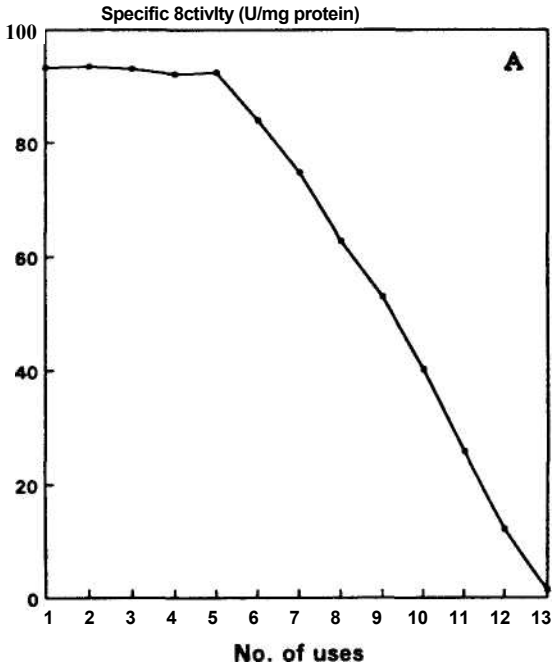
In gelatin immobilization, the specific activity of the immobilized enzyme was found to be only 63% of the activity exhibited by the free enzyme. The protein nature of gelatin, its high hydrophilicity and consequently, its strong swelling power provide environmental conditions very favourable for immobilized enzymes and reduce the mass-transfer resistance to the diffusion of substrate and reaction product [30]. The hardening treatment with formaldehyde to achieve thermostability and mechanical strength renders it less active. However, the flat membranes were stored refrigerated (4°C) for nearly 2 months without any appreciable loss of enzyme activity (63% of the free enzyme activity) in deionized water **(Fig. 6).**

Table 8 gives a comparison of the two different matrices used for immobilizing SOD. DEAE Sephadex A-50 was found to be an easy and efficient matrix for immobilizing SOD with a good specific activity. Gelatin also is an inexpensive, abundant and safe material, largely used as a food additive and the problem of enzyme inactivation by formaldehyde needs to be solved by trying other treatments for hardening. These preliminary results on immobilization of SOD from Spirulina would be an addition to the industrial applications of SOD.

One of the applications of Spirulina in pharmaceutical industry is its ability to quench free radicals in human system due to SOD, thereby reducing health risks and retarding ageing process [31]. For large-scale production of enzymes, it is often favourable to choose enzymes of microbial origin. Enzymes produced for use in food are preferably obtained from microorganisms such as yeasts, lactic acid bacteria, etc. which are traditionally being used in food technology.

Figure 6A. Specific activity of SOD immobilized in Sephadex A-50 on repeated usage.

68. Storage stability of SOD immobilized on gelatin



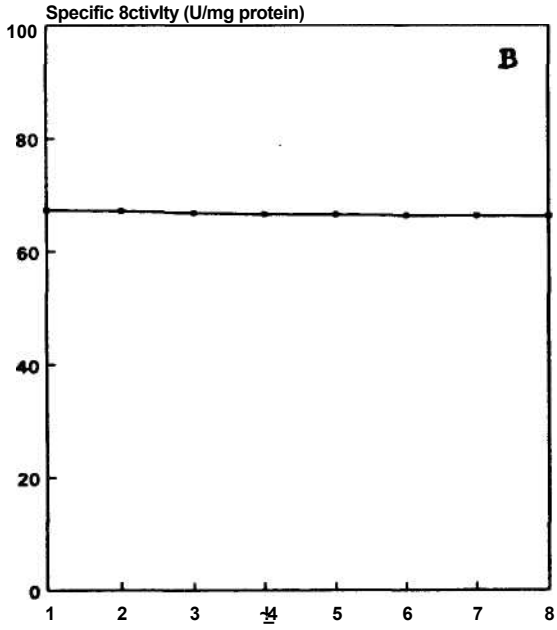


Table 8 : A comparison of the two different matrices used for immobilizing SOD

S.No.	Matrix	Enzyme to matrix ratio	Specific activity	No. of uses	Storage stability
	DEAE Sephadex A-50	**1 : 16**	88% of the original activity	No loss for 5 uses after which, 10-15% loss per usage	Stable at 4°C
	Gelatin	1 : 10	63% of the original activity	No appreciable loss in activity	Stable at 4°C

Purified SOD from Spirulina was found to act as an antioxidant in the oxidation system of emulsified linoleic acid. The origin of the enzyme from a food grade organism and its particular properties render it attractive for use in food industry. It is moderately stable to heat and hence could be incorporated into food materials or beverages that need no heavy heat treatment. The low cost of production and ready availability of the source material may also be advantageous to large scale purification work.

EXPERIMENTAL

Chemicals used

Triple distilled and deionized water was used throughout. Sephadex G 50-150, NBT, acrylamide, bis-acrylamide, standard proteins for molecular weight determination, catalase, bovine SOD, gelatin and DEAE Sephadex A-50 were purchased from Sigma Chemicals & Co. Riboflavin and methionine were purchased from Sisco Research Laboratories Pvt. Ltd., Bombay. All other chemicals used were of the highest purity available locally.

Organism and culture conditions

Clonal and axenic culture of Spirulina maxima (FTCC 03) was used for the study. The culture was grown in Zarrouk's liquid medium [32] in 1L shake flasks at a light intensity of 100-150 $\mu E\ m^{-2}\ s^{-1}$, temperature of 32°C and an agitation of 90 rpm on a rotary shaker.

Purification

Cells from the culture of Spirulina maxima (FTCC 03) in the mid logarithmic phase (0.0. at 560 -1.2) were collected by centrifugation at 5,000 g for 20 min. The entire purification procedure was carried out at 8-10°C. The pelleted cells were washed twice and suspended in 0.2 M phosphate buffer, pH 7.8. The suspension was sonicated at 10 K cycle/sec (ultrasonication) for 1 min to disrupt the cells. The supernatant collected after centrifugation at

10,000 g for 30 min served as the crude enzyme extract. This was subjected to 55-85% ammonium sulfate fractionation. The resulting fraction, after dialysis against 0.015 M phosphate buffer, pH 7.8, was loaded onto DEAE Sephadex A-50 ion-exchange column (3.6 x 40 cm) equilibrated with 0.015 M phosphate buffer, pH 7.8. The elution was carried out with increasing strengths of phosphate buffer (0.015, 0.02, 0.03, 0.04 and 0.05 M). The enzyme got eluted in 0.05 M phosphate buffer. The active fractions were pooled, concentrated, dialyzed in 0.015 M phosphate buffer, pH 7.8 and applied to a column of Sephadex G 50-150 (1.6 x 140 cm), equilibrated with the same buffer. The flow rate was 0.32 ml/min. The void volume was 35.0 mI. The volume of the fractions collected was 1.5 mI. The fraction with the enzyme activity eluted after approximately 47 ml (excluding void volume). They were collected, pooled and concentrated.

Enzyme assay

SOD activity was assayed photochemically. The original assay described by Beauchamp and Fridovich (1971) [33] was suitably modified. The reaction mixture (pH 8.0) consisted of 1.3μM riboflavin, 13 mM methionine, 63μM NBT and an appropriate volume of the enzyme. Distilled water was added to bring to the final volume to 3 mI. The assay mixture was illuminated in glass test tubes selected for uniform thickness and colour. Identical solutions that were not illuminated served as blanks.

The apparatus devised for exposing the tubes to light consisted of a test tube holder, immersed in water in a glass container thermostated at 25°C. Two circular fluorescent lamps (giving a light intensity of 4,000 lux) were attached to the water bath from outside and the entire assembly was fitted in a box lined with aluminium foil. The reaction was initiated and terminated by turning the lights on and off [34].

The initial rate of reaction was determined as increase in absorbance at 560 nm. Under the conditions described, the increase in absorbance in the absence of SOD was 0.1 0.015 min which was linear upto 15 min. In the presence of SOD, the reaction was inhibited and the amount of inhibition was

used to quantitate the enzyme. Each sample was assayed twice and the results varied less than _+ 0.005 O.D. unit/5 min.

SOD units/ml = [(V/v)-1] (dilution factor) [34]

where V and v represent the rate of the assay reaction in absence and in presence of SOD, respectively. The protein content was estimated using Lowry's method [35] to determine the specific activity of the enzyme (Activity in Units/mg protein).

Polyacrylamide gel electrophoresis

Non-dissociating, high pH, discontinuous polyacrylamide gels were prepared using 7.5% gel for separation and 3.5% gel for stacking in a mini slab gel system (dimensions: 15x18x15, vertical; Bangalore Genei Pvt. Ltd.). The gels were stained for SOD activity by the method of Beauchamp and Fridovich (1971) [33] and for proteins by silver staining [9].

Superoxide dismutase was localized by soaking the gels in 2.45x10-3 M NBT for 20 min, followed by an immersion for *15* min, in a solution containing 0.028 M tetramethylethylenediamine, $2.8x10^{-5}$ M riboflavin, and 0.036 M potassium phosphate at pH 7.8. The gels were then placed on a dry glass plate and illuminated for 5 to 15 min. During illumination, the gel became uniformly blue except at position containing superoxide dismutase. Illumination was discontinued when maximum contrast between the achromatic zones and the general blue colour had been achieved [33].

The molecular weight of protein subunits were determined in SDS-PAGE using the following markers; carbonic anhydrase - 29,000; trypsinogen - 24,000; p-lactoglobulin - 18,400 and lysozyme - 14,300. Protein bands were detected by silver staining.

Molecular weight determination

The relative molecular mass of the enzyme was determined by gel-permeation chromatography using the column of Sephadex G 50-150 (with the same

conditions as described above for purification). The standard proteins used were egg albumin - 45,000; carbonic anhydrase - 29,000; trypsinogen - 24,000; I-lactoglobulin - 18,400 and lysozyme - 14,300. The enzyme was detected by the measurement of the activity as described above and standard proteins were detected by measuring the absorbance at 280 nm.

Effect of pH

The effect of pH was determined by diluting SOD in a number of buffers, of 15 mM) in a pH range of 3 -9 [citrate buffer (pH 3.0-4.0), sodium acetate buffer (pH 4.0-5.6), phosphate buffer (pH 5.7-7.2) and Tris-CI buffer (pH 7.0-9.0)]. The enzyme suitably diluted was used for the assay.

Temperature optimum and temperature stability

The optimum temperature for the enzyme was determined by carrying out the assay at various temperatures ranging from 4°c - 30°C (4, 5, 10, 15, 20, 30°C).

For finding out the temperature stability of the enzyme, aliquots of SOD were incubated in 15 mM phosphate buffer, pH 7.8, at a series of elevated temperatures (4°C, 10-80°C with a 10°C raise for each in the range) for 30 min before cooling in ice and assaying for SOD activity.

Atomic Absorption Spectrophotometry

The purified enzyme preparation (about 1 mg) in buffer (0.015 M phosphate buffer, pH 7.8) was taken in a silica crucible. The crucible was placed in a drying oven at 105°C for about 2 hrs. Charred under low flame until smoke ceased and ashed at about 400°C in a muffle furnace until the sample became carbon free. Dissolved the ash under a watch glass with 5 ml of HC1. Added 20 ml of distilled water and evaporated to near dryness on a steam bath. Added 20 ml of 0.1 N HCI and continued heating for a few minutes. Carefully rinsed the watch glass into the crucible and quantitatively transferred the contents through a Whatman filter paper no. 41 into a 100-ml

volumetric flask. Washed the crucible with several portions of 0.1 N HCI, transferring each to the volumetric flask, and made up the volume with 0.1 N HCI. Duplicate 5-ml portions of 0.1 N HCl were carried out throughout the procedure for blanks. Iron, zinc, copper and manganese were quantitatively determined from these solutions using atomic absorption spectrophotometry (Perkin Elmer 3110) [36]. The protein content in the sample used was determined using Lowry's method [35].

Antioxidative effect in Linoleic acid model system

The antioxidative effect of SOD in an emulsified linoleic acid model system was measured by spectrophotometrically as described by Lingnert ~ 2l. [37].

The enzyme extract was mixed with 2 ml of 10 mM linoleic acid emulsion, pH 6.5 in test tubes (emulsified with the aid of an equal amount of Tween 20 in 0.1 M potassium phosphate buffer). The tubes were placed in dark at 37°c to accelerate the oxidation. Controls without antioxidant were run parallel. Both before starting the incubation and after 36 hr, 0.2 ml of the substrate-antioxidant mixture was withdrawn and solubilized in 2 ml of absolute, spectral grade methanol. Then 6 ml of 60% methanol in water was added and the absorption measured at 234 nm [37].

The antioxidative effect (A.E.) was calculated according to the following equation.

$$A.E. = \frac{(C) - A_{234}}{A\ A_{234}\ (C)}$$

(A A234 is the increase of absorption at 234 nm during the incubation time is the corresponding increase in the control).

The A.E. was expressed in values ranging from 0 to 1, where 0 represents no antioxidative effect and 1, complete inhibition of the oxidation. (Negative values indicate a prooxidative effect).

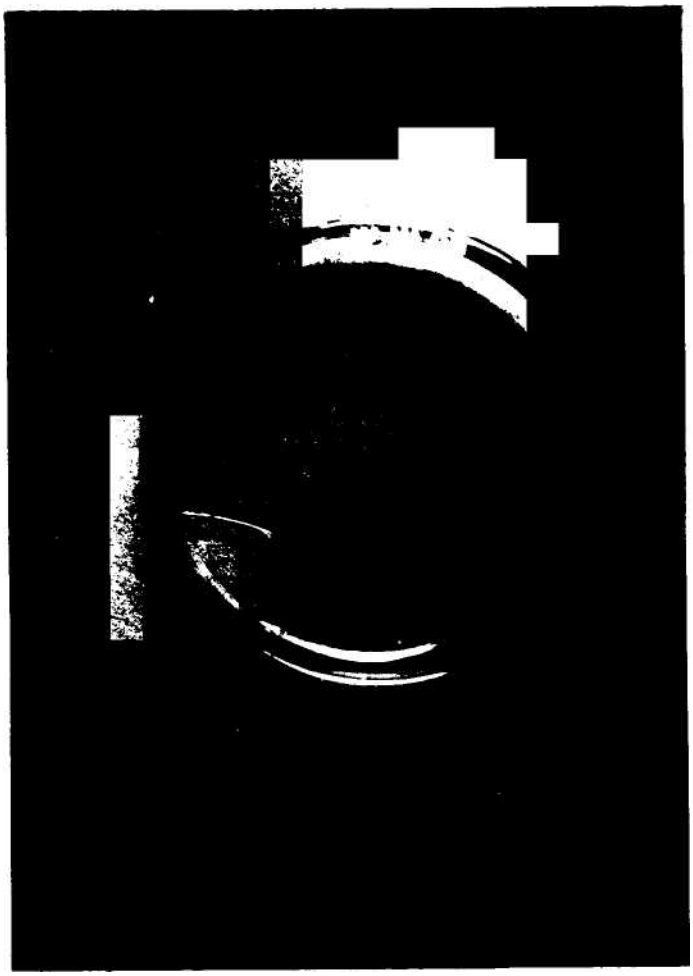
Immobilization of SOD

The purified enzyme (500 J1L) preparation (1 mg protein/ml) was immobilized onto DEAE Sephadex A-50 equilibrated with 0.015 M phosphate buffer, pH 7.8, to give a final enzyme to matrix ratio of 1:16. The matrix (with the bound enzyme) was washed thoroughly to remove the unbound free enzyme. The enzyme assay was performed as given above, taking an aliquot of the matrix representing approximately 50 μg of the enzyme.

Immobilization in gelatin was done using the method reported in reference [30]. To 9.5 ml of an aqueous solution of 10% (w/v) gelatin at 35-40°C, added *0.5* ml of a hardening solution consisting of 20% (w/v) formaldehyde in *50%* (v/v) ethanol. Within 1 min, an appropriate volume of enzyme solution (1 mg protein/ml) was mixed to give a final enzyme-to-gelatin ratio of 1:10. 10 ml of the gelatin mixture was spread as uniformly as possible in a petri plate. The plate was quickly put into a deep freezer (about -25°C) and after 5 hrs was gradually brought to room temperature (Fig. 7). The thin membrane (-0.1 cm) thus formed, once removed from the petri plate was thoroughly rinsed with water and stored in a refrigerator (4°C) in deionized water.

Figure 7. Purified SOD immobilized on gelatin membrane

—> - indicates the portion of the membrane withdrawn for the enzyme assay corresponding to *50* µg of the enzyme



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CHAPTER 5

ATTEMPTS ON GENETIC IMPROVEMENT OF *SPIRULIINA* FOR VALUE ADDITION

SUMMARY

A method was developed for the isolation of protoplasts from Spirulina platensis. Trichomes were treated with lysozyme (0.1%) for 28 hrs at room temperature. This resulted in 80% yield of protoplasts. Eventhough the cells were intact in their fine structure and viable as evidenced by viability assay, they were not regenerable. Hence, another rapid method was developed for the production and regeneration of protoplasts, where the protoplast isolation could be achieved in about an hour, with a good regeneration efficiency. The latter method is based on the removal of the outer sheath covering the trichomes by pre-incubation wash with NaCI to render the cell-wall amenable for quicker digestion with lysozyme. This required a lower concentration of 0.01% of lysozyme. A novel viability assay was standardized using a dual dye indicator, involving fluorescein diacetate and calcofluor white. Later, it was found that the viable cells autofluoresce red. Hence, calcofluor white alone was effective to differentiate the viable from the non-viable cells.

Also, attempts were made to transform Spirulina with bacterial plasmid pBR322 using intact trichomes as well as prepared protoplasts by direct DNA uptake. It appeared that Spirulina is transformable, by the resistance shown by the cells treated with plasmid DNA to ampicillin or tetracycline, while the normal, untreated cells did not grow in the presence of these antibiotics.

Very little has been done by genetic means to improve the strains of cyanobacteria presently utilized for biotechnological purposes. But, genetics has played a major role in the development of commercially important strains of other bacteria. Not a single ocean bacterium or alga has been dissected to the same molecular level as E.coli. In fact, only in the last few years, have researchers successfully developed protoplasts from a few selected seaweeds. Regenerating these protoplasts, however has proved to be a formidable task. Thus, protoplast fusion, classical breeding and genetic manipulation are at roughly the same nascent stage of development.

The immediate technical challenge in cyanobacterium is to introduce a new gene trait or obtain higher levels of nutritionally or industrially important components (e.g. protein, amino acids, certain lipids, pigments, vitamins, enzymes like SOD etc.).

Any attempt to genetically manipulate these organisms depends on the availability of the systems allowing the transfer and the expression of foreign genes in the cells. So far, no plasmid or phage infecting Spirulina has been isolated. Moreover, the filamentous nature of the organism and its non-colony formation have resulted in very little work on genetic manipulation of this alga [1].

In the absence of sexual reproduction, preparation of protoplasts followed by regeneration is advantageous for genetic manipulation through direct DNA-uptake or protoplast fusion. Very few reports exist on successful isolation of protoplasts from blue-green algae. Biggins (1967) prepared metabolically active protoplasts from Phormidium luridum [2]. Both spheroplasts [3] and protoplasts [4] have been isolated from Anabaena. Robinson *et* al (1982) prepared only spheroplasts from Spirulina to provide an alternative method for studying electron transfer activity [5].

This is the first successful report on isolation of protoplasts from Spirulina, followed by their regeneration. Regenerating protoplasts to normal trichomes may offer an interesting tool for introducing desirable traits into

the strains. Here, attempts were also made for direct transformation of Spirulina with the bacterial plasmid pBR 322, using both intact trichomes as well as prepared protoplasts of Spirulina.

RESULTS AND DISCUSSION

At the indicated growth points (stages) shown in Figure 1, trichomes were harvested and used for protoplast preparation.

Protoplast formation, purification and viability (Method 1)

Figure 2 shows phase contrast photomicrographs of an untreated filament of Spirulina platensis and enzymatically treated cells at various stages of protoplast preparation (Fig. 2 A **to** D). The sheath got removed after 20 hrs of incubation and long strings of cells held together at the cross walls were formed **(Fig.** 2 B *&*. D). Gentle swirling of the incubation mixture resulted in the formation of single cells **(Fig.** 2D) which were readily separated from the long undissoclated filaments on sucrose gradient centrifugation. This resulted in an approximately 80% yield **(Fig. 3** and **Table 1),** as determined by counting the released protoplasts.

A technique was developed earlier in our lab, involving dual dye indicators for checking the viability of the trichomes and protoplasts [6]. The dyes used were fluorescein diacetate and calcofluor white which fluoresce red and blue, respectively in the UV region on excitation. But later, it was noticed that the viable Spirulina cells exhibited autofluorescence (red colour). Hence, the dye fluorescein diacetate was eliminated and calcofluor white alone was used to differentiate the viable from the non-viable cells.

Calcofluor white acts specifically on cellulose giving a bright blue fluorescence in the UV region. This property was employed in testing the absence of cell-wall material in the isolated protoplasts of higher plants [7] and in protoplast culture for checking the cell-wall resynthesis. Here, the Spirulina cells fluoresced (auto fluorescence) bright red, in the same region

Figure 1. Growth curve of S.platensis

The growth was monitored by A^Q. Cells obtained at different stages were analyzed fo,r: protoplast release, purification, viability and regeneration

A - stages used for protoplasting studies o
- stages not used for the studies

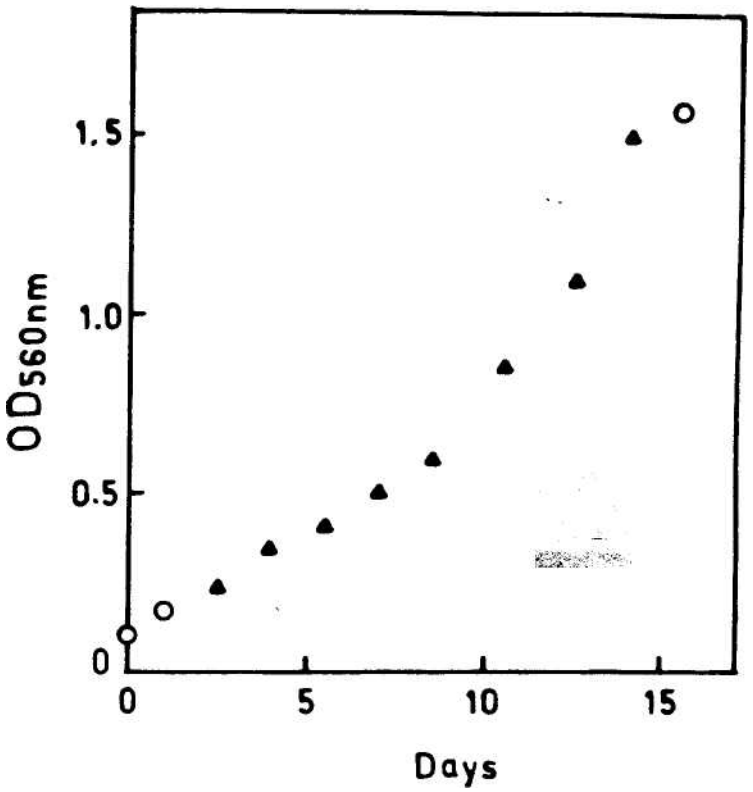
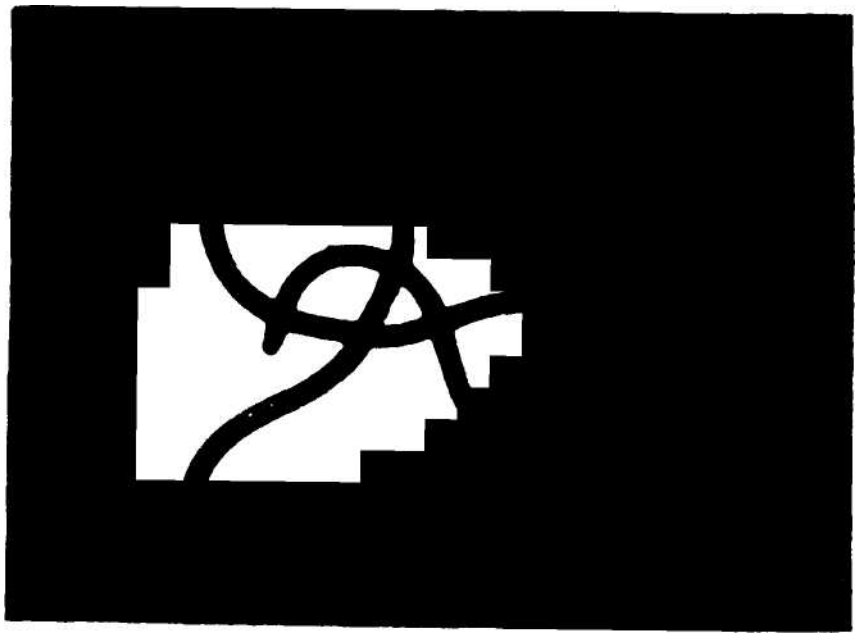


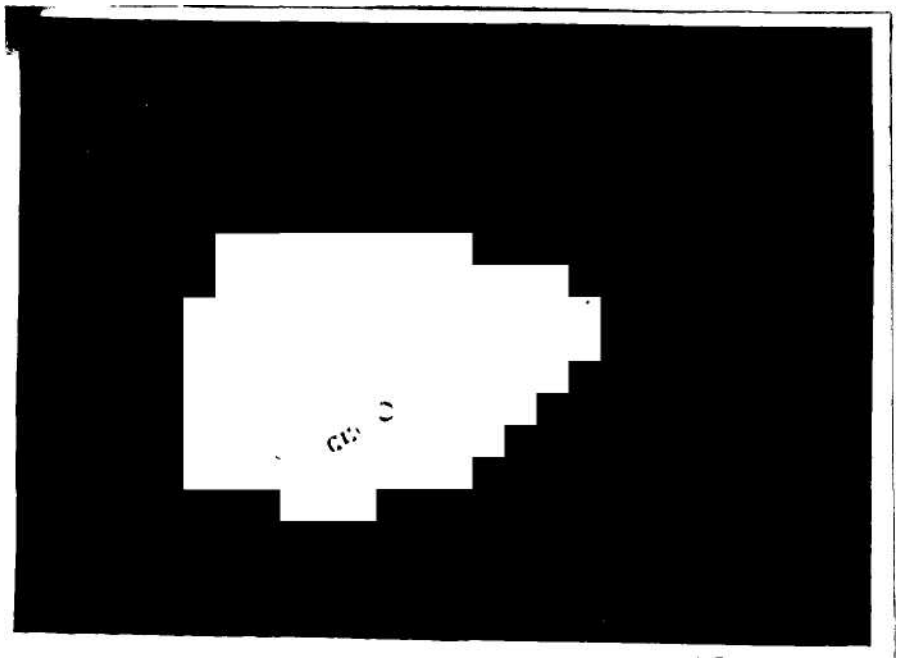
Figure 2 Microscopic observation of different stages of prOtOplast preparation from S.platensis (Method 1) (magnification: 670 **x)**

A) Untreated trichome



B) Cell-wall digestion of the trichomes

C) Protoplast preparation before purifying on sucrose gradient



D) Purified protoplasts

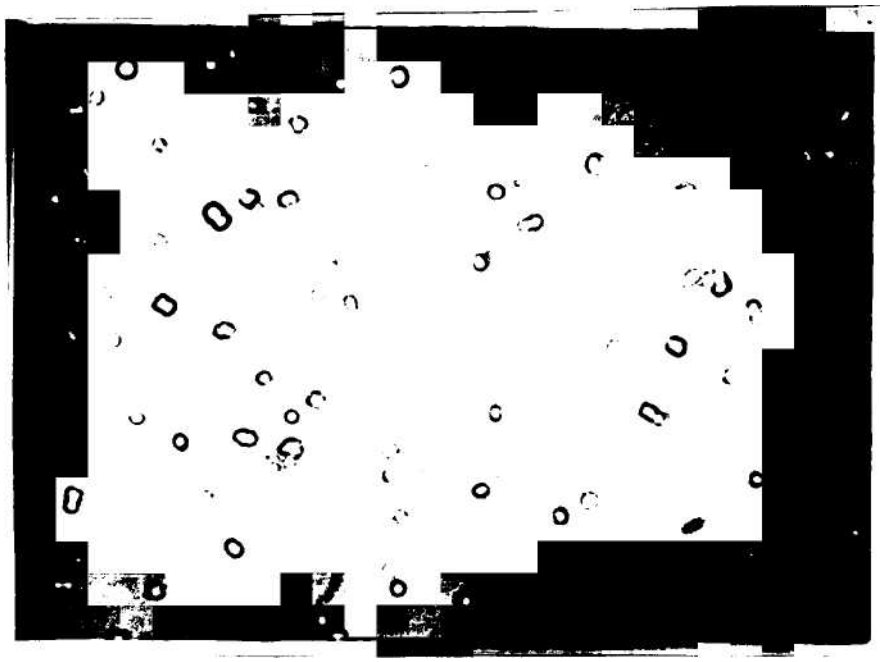


Figure 3. The percentage yield of protoplasts

Protoplast yield from S.platensis as a function of the growth stage. Trichomes obtained from the indicated A^g were treated for protoplast release (©—©) Method No. 1 (•—•) Method No. 2

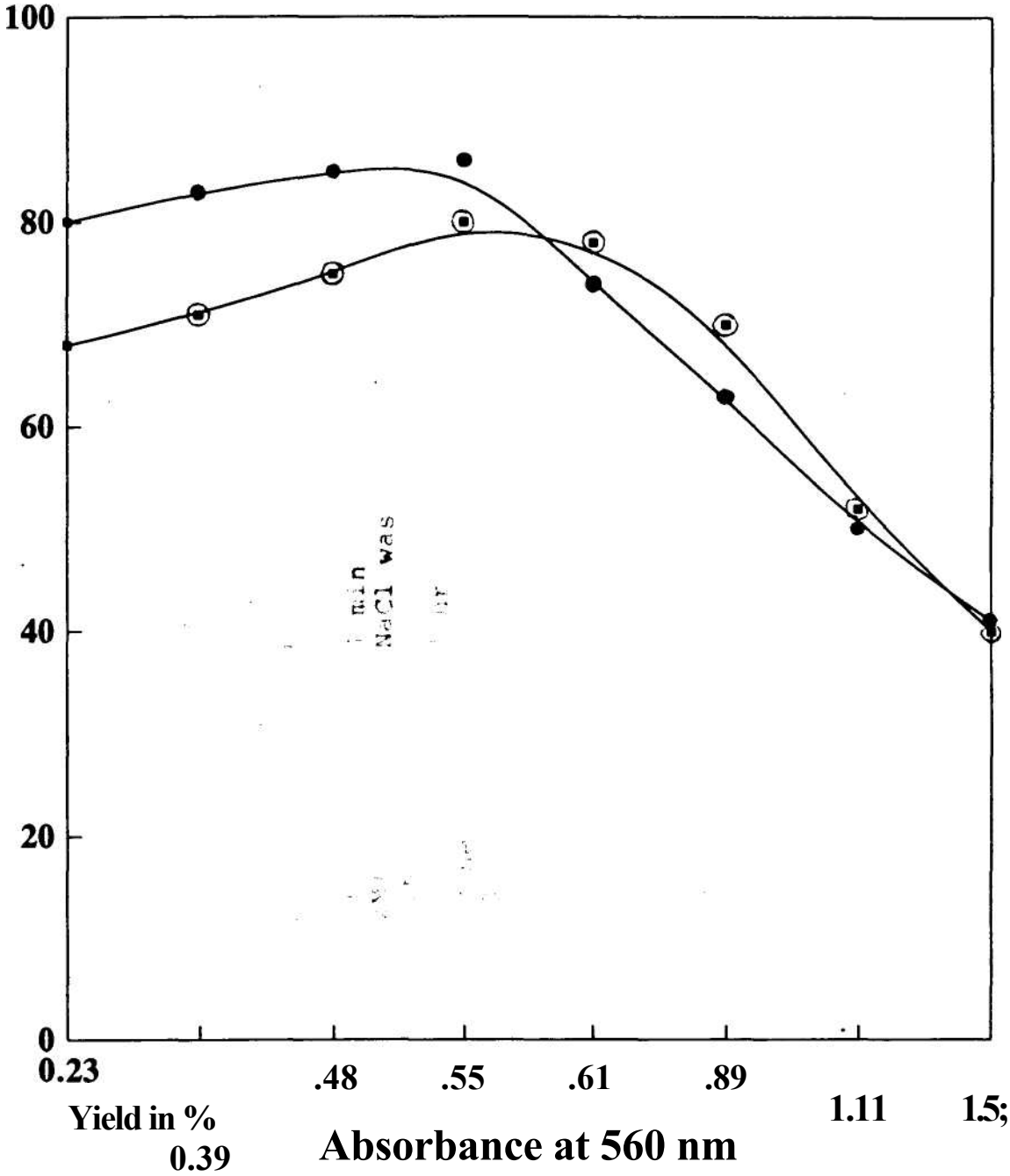


Table 1 : Comparison of the two different methods of protoplast preparation

Method	Medium	Lysozyme cone,	Incub. time	Cells # Stability lity	Yield released	Viabi (%) -	Regenerati
	0.5 M Manmtol-0.03 M Phosphate (pH 6.8)	0.1%	28 hrs	Proto-plasts	80	Stable	Not regene -able only for 4-6 hrs
	2 mM EDTA + lysozyme				41		
2A	1.5 M NaCl	Nil	1 min	Sphero	86	Quite stable	Regenerate in regenera-to trichom -tion medium
	Sol. P : {10 mM Tricine -NaOH; pH 8; 2 mM $MgCl_2$ 2 mM $CaCl_2$ 40 mM NaCI 10% PEG)		1 hr		49		
2B	Same as 2A + enzyme	0.01%	1 min NaCl wash	Proto-plasts	86	Quite stable in medium	ated to trichomes (Table 2)
	Sol. P + lysozyme		1 hr		49		

Spheroplasts are single cells in which the cell wall is not digested completely.
ZM - Zarrouk's medium

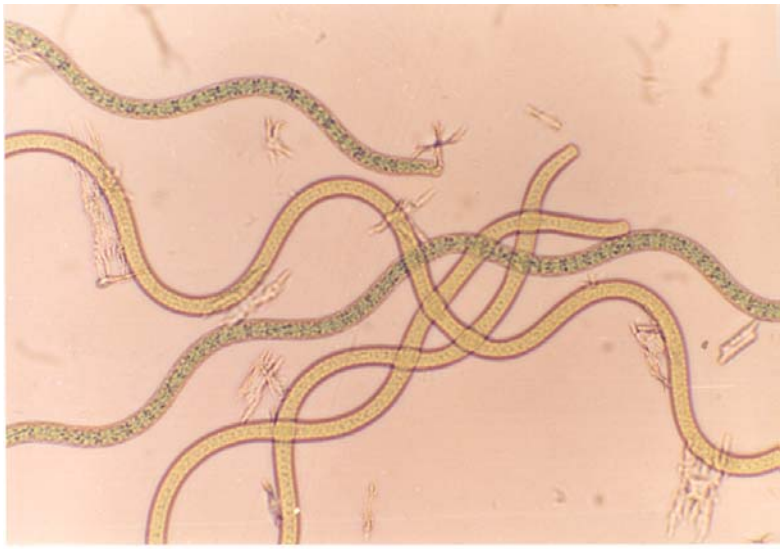
due to the excitation of phycobiliproteins **(Fig.** 4B). Thus, when calcofluor white was added to the protoplast preparation, viable cells fluoresced bright red and dead ones blue (Fig. 40). The blue fluorescence in dead cells is due to the entry of calcofluor white into the non-viable cells following the dye-exclusion principle of the conventional dyes. It is not due to dye-binding to cellulose, as cellulose is absent in the cyanobacterial cell wall. **Figure 4E** shows a single viable protoplast fluorescing red in the UV region. The conventional method of dye-exclusion using Evan's blue staining is shown in **Figure 4F.** Though calcofluor white acts both on viable and dead cells, the fluorescence caused by this in viable cells was masked by the bright red fluorescence. Blue fluorescence was therefore seen only in non-viable cells. In the visible range **(Fig.** 4 A &: C), viable cells appeared green, while non-viable cells lacked the green colour due to loss of chlorophyll and other pigments.

The maximum percentage of viable protoplasts obtained by this method is 41% and the percentage of viable protoplasts increased with the age of the culture **(Fig.** 5). It was found that the protoplasts washed and maintained in buffer were stable for 4-5 hrs if stored at 4°C. The isolated cells were spherical and intact in their fine structure. They burst readily on addition of distilled water due to the change in osmotic pressure.

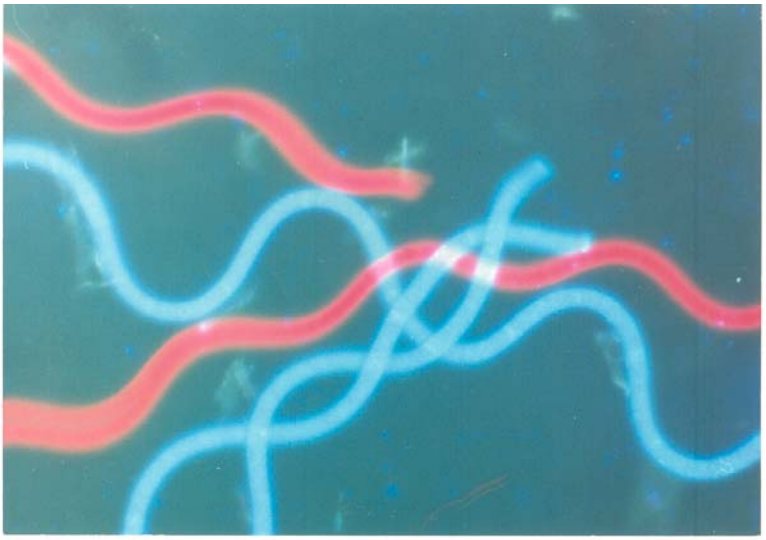
Figure 6 shows the absorption curves of an intact trichome suspension of Spirulina **(Fig.** 6A) and a cell suspension treated with lysozyme **(Fig.** 6B). A fall in absorbance was noted which indicated the release of protoplasts. The optimum concentration of mannitol was deduced by measuring the relative contents of phycocyanin in the supernatant after treatment with enzyme solutions **(Fig.** 7), as phycocyanin release is an indication of cell lysis. It was found that the incubation time for enzyme digestion is directly proportional to the cell density. The incubation time decreased to 3-5 hrs when the trichomes were sonicated before enzyme treatment, but this led to an extensive damage of the cells resulting in heavy loss of phycocyanins.

Figure 4. Viability of dead and viable trichomes and protoplasts of S. platensis (magnification: 670 x)

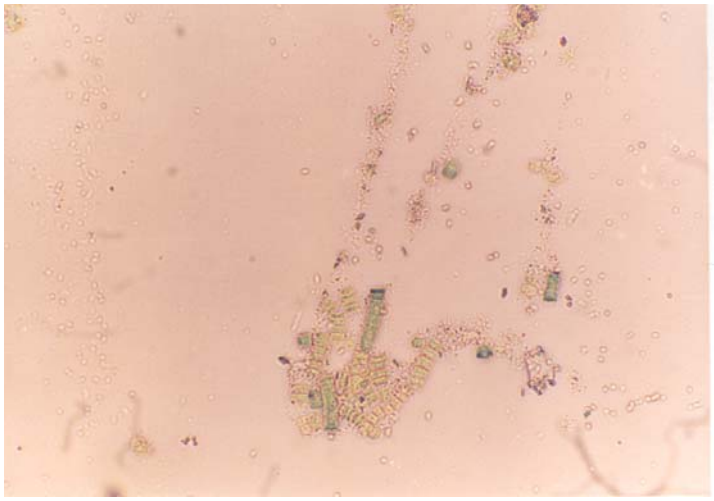
A) Mixture of dead and viable trichomes in the visible range (green- viable; yellow- non-viable)



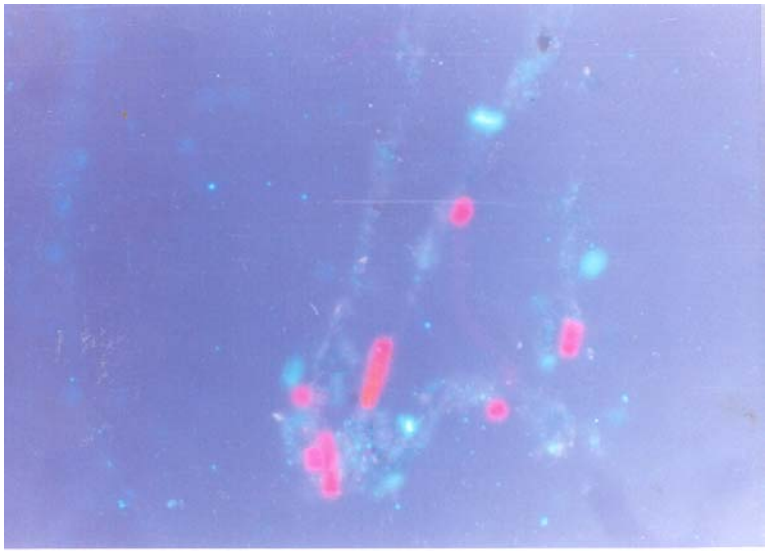
B) Mixture of dead and viable trichomes in the UV range (red-viable; blue- non-viable)



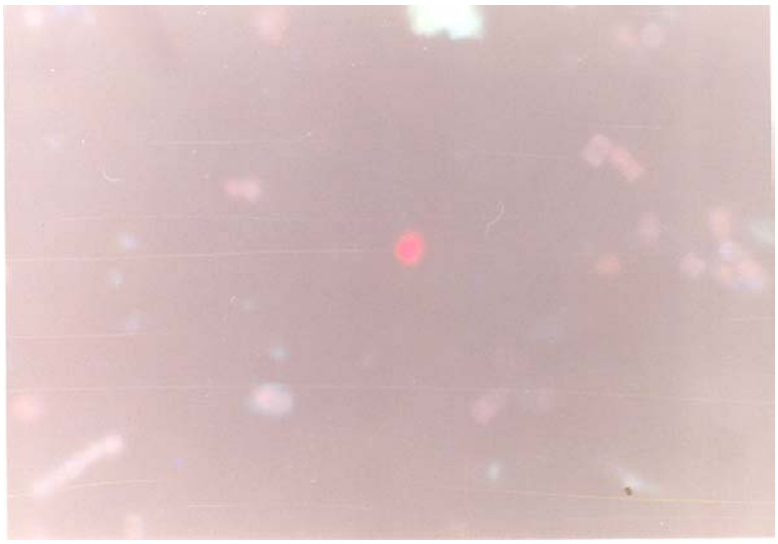
C) Mixture of dead and viable cells in the visible range during protoplast preparation



D) Mixture of dead and viable cells in the UV range during protoplast preparation



E) A viable protoplast in the UV region



F) Viability test using Evan's blue vital staining (cells which exclude the dye are viable)

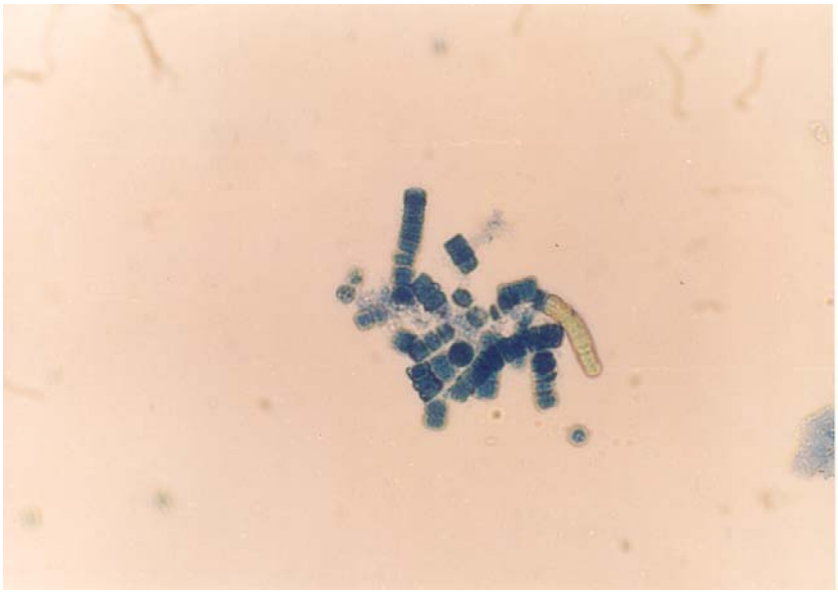


Figure.5. The percentage viability of protoplasts

Protoplasts obtained from the various growth stages of S.platensis (A_{560}) were checked for viability

(o-o) Method No.1(●-●) Method No.2

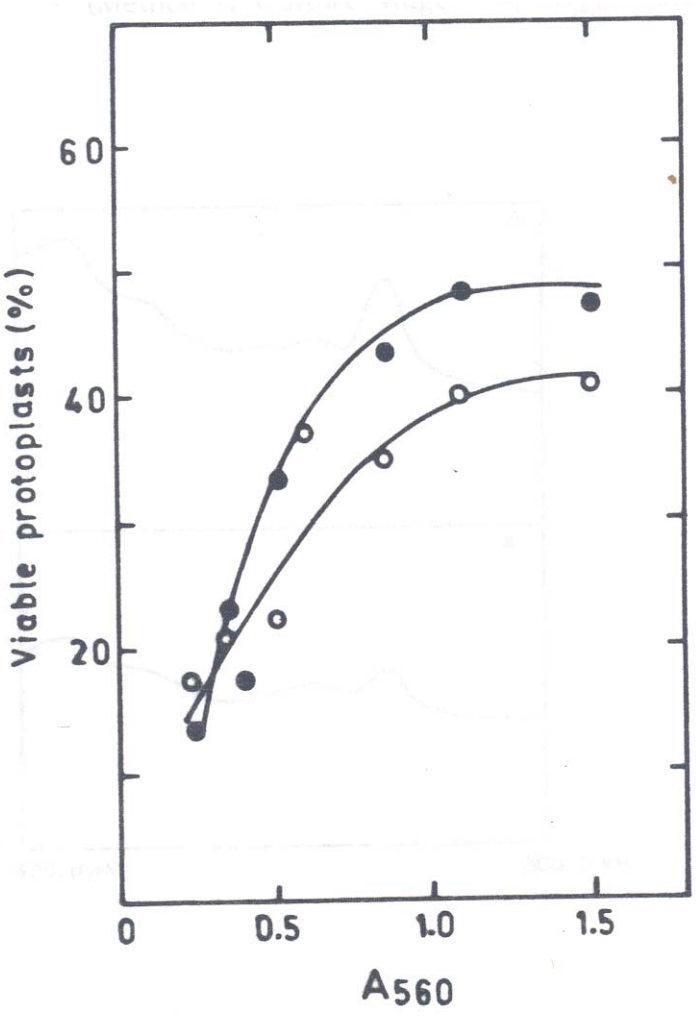


Figure 6. Absorption curve of

A) intact trichome suspension

B) protoplast suspension at the end of the treatment (Method 1; Culture stage - 4; A560 -0.55)

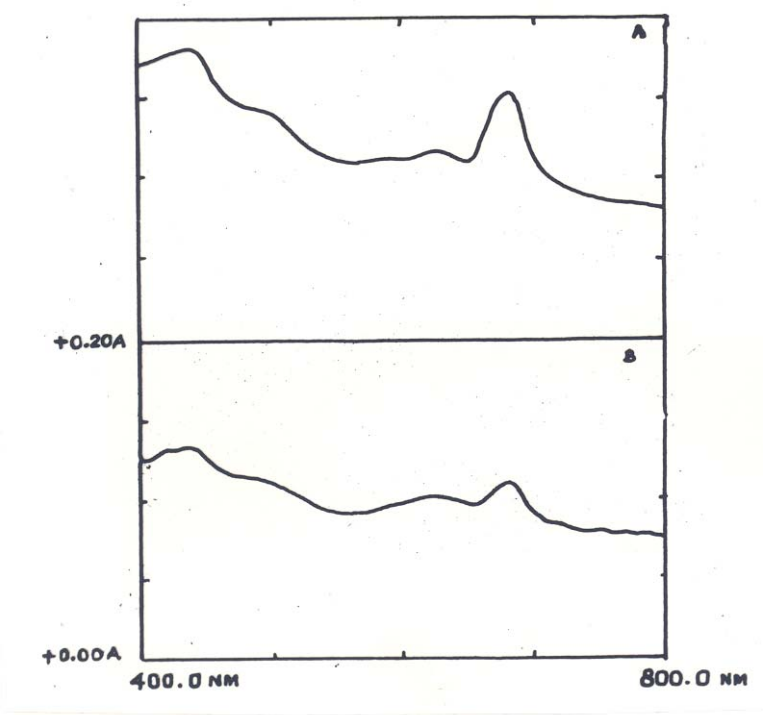
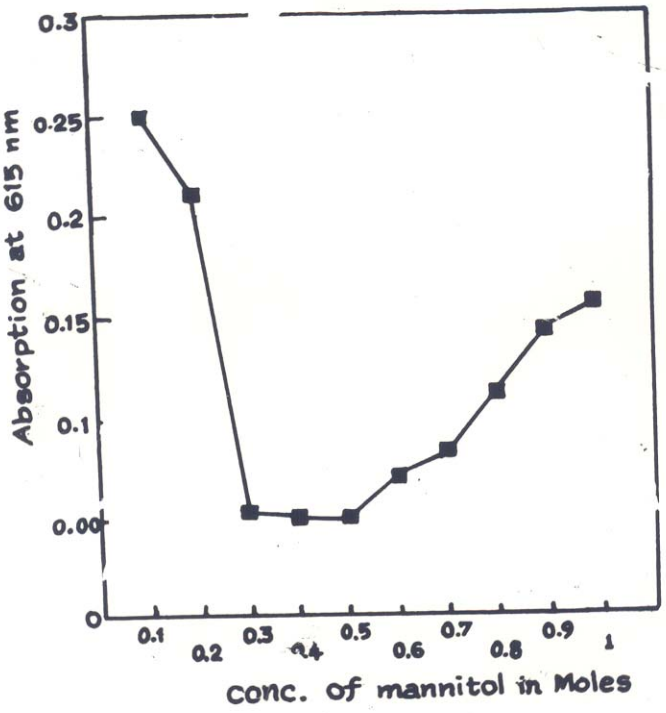


Figure 7. Test for intactness of the protoplasts released during protoplasting (To optimize the mannitol concentration)

A_{615} was monitored at different osmotic levels (mannitol concentrations); A_{615} indicates the release of phycocyanin due to cell injury

(Method 1; Culture stage -4; A560 -0.55)



Protoplast formation, purification and viability (Method 2)

The previous method had the following disadvantages

* The time needed for protoplast preparation is too long (28 hrs).
* The protoplasts were viable only for 4-5 hrs at 40c.
* They were not regenerable.

Hence another rapid method was followed for protoplast isolation.

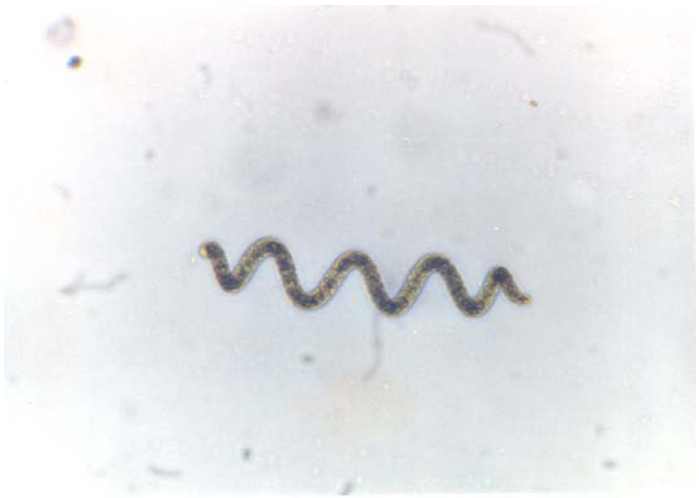
Due to the presence of an outer sheath, the trichomes of S.platensis are resistant to lysozyme (upto 2 mg/ml). To remove the outer sheath and thus render the cell wall sensitive to lysozyme, Robinson et al: [5] washed the trichomes with EDTA and KC1. To obtain protoplasts capable of regeneration, this procedure was modified by eliminating the EDTA wash and the fragmentation of the trichomes. NaCl (1.5 M) was substituted for KCl and the length of exposure to NaCl was standardized. The exposure time was kept minimum so that the normal growth cycle resumed to reestablish the trichomes under standard conditions. **Figures 8 A to C** show the photomicrographs of the different stages of protoplast preparation using this method.

The incubation of the trichomes in Sol. P without lysozyme (after NaClwash) resulted in only spheroplasts which are single cells in which the cellwall is not completely digested (Fig. 9). Incubation of the NaCl-washed .trichomes in Sol. P in the presence of 0.01 % lysozyme gave rise to true protoplasts. The yield was nearly 86% (Fig. 3 and Table 1) as determined by counting the released protoplasts.

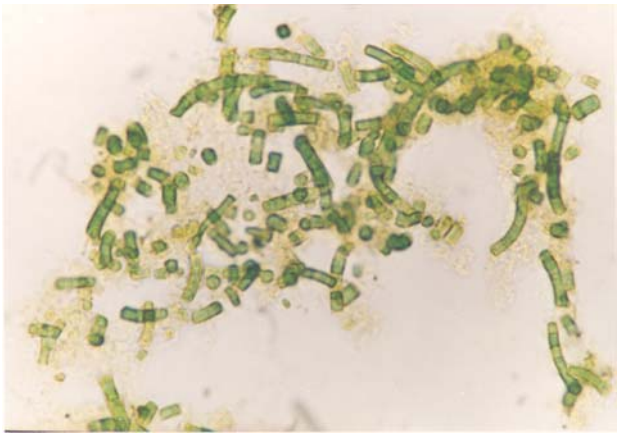
The protoplasts formed were able to regenerate (Fig. 10 A &: B). This could mean that, cells can be detached from one another and the wall is either naturally discontinuous or removed by the high salt treatment upon removal of the outer sheath of the trichomes. Regardless of exposure of the trichomes to lysozyme, the length of the treatment necessary to obtain protoplast regeneration varied very much depending on the age of the trichomes. In

Figure 8. Microscopic observation of the various stages of protoplast preparation from S. platerisis (Method 2) (magnification: 670 x)

A) A normal trichome



B) Cells after 30 min treatment in Sol.P



C) Protoplast preparation purified on the albumin gradient

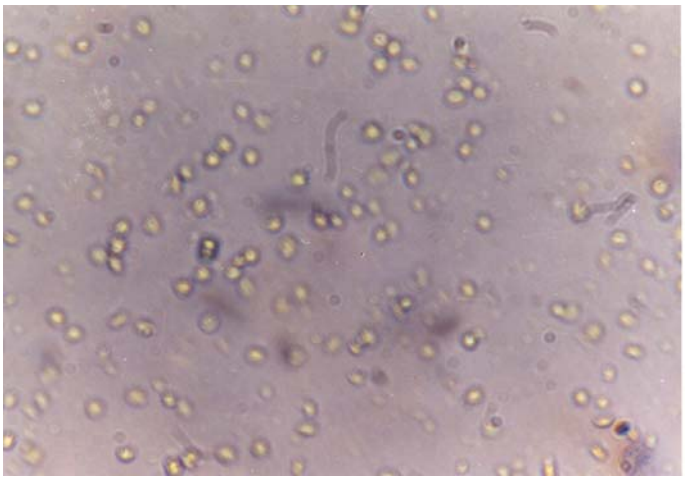
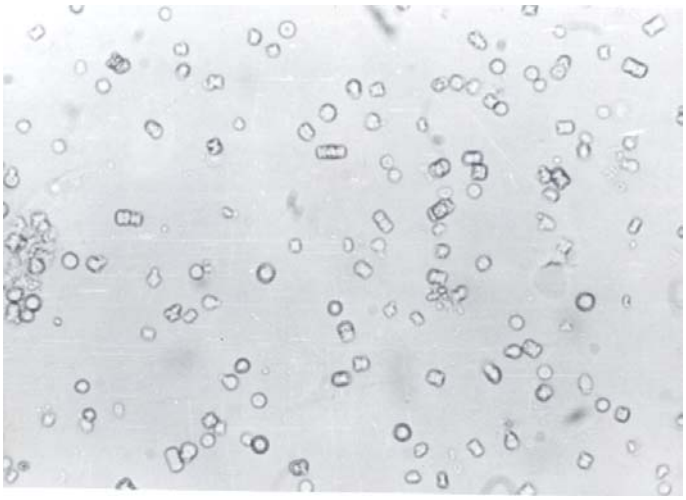


Figure 9. Photomicrographs of spheroplasts (magnification: 670x)

The single cells released on treatment of the Spirulina trichomes in Sol. P in the absence of lysozyme



general, shorter times were required for younger (60-70 min) cells compared to older cells. Longer treatments allowed a better fragmentation of the trichomes but yielded a lower amount of viable protoplasts **(Fig. .5)**.

Following fragmentation of the trichomes, it was necessary to separate the protoplasts and single cells present from the partially fragmented and intact trichomes. Discontinuous gradients of albumin gave a good separation and the concentration used was harmless for both protoplasts and untreated trichomes. Due to the fragility of the protoplasts, it was important that the centrifugal force was kept low, while the centrifugation time had to be varied (between 10 and 45 min) depending on the age of the trichomes used. Older cells required longer centrifugation times. A good separation resulted in the appearance of a single band in the upper fractions of the gradients, with partially fragmented or unfragmented trichomes pelleting at the side of the tube. The fractions containing protoplasts were never found to contain trichomes and only occasionally cell doublets were seen under the microscope. These doublets likely correspond to cells which were dividing within the trichome at the time of the treatment.

The protoplasts were obtained from trichomes harvested at eight different time points during growth, (Fig. 1). The viability of the protoplasts following treatment in Sol. P is shown in Fig..5. The viable protoplasts were recognized by Evans blue vital staining and fluorescence as described above (Fig. 4 A to E). The percentage of viable protoplasts obtained by this method was nearly 4996 (Fig. .5). It increased with the age of the culture. The protoplast preparation was found to be stable in regeneration medium at normal growth conditions.

The main advantage of this method over the previous method described earlier (Method 1) is that, the cells responded to a very low concentration (as low as 0.0196) of lysozyme and resulted in intact protoplasts in good yield with the capacity to regenerate. This indicates that the pre-incubation wash of 5pirulina trichomes with NaCI is very effective in rendering the cells amenable for quicker digestion of cell-wall to release the protoplasts. Also the yield and viability of the protoplasts were better for Method 2 as compared to Method 1. **Table 1** gives a comparison of the two different methods used for protoplast preparation.

Regeneration of protoplasts

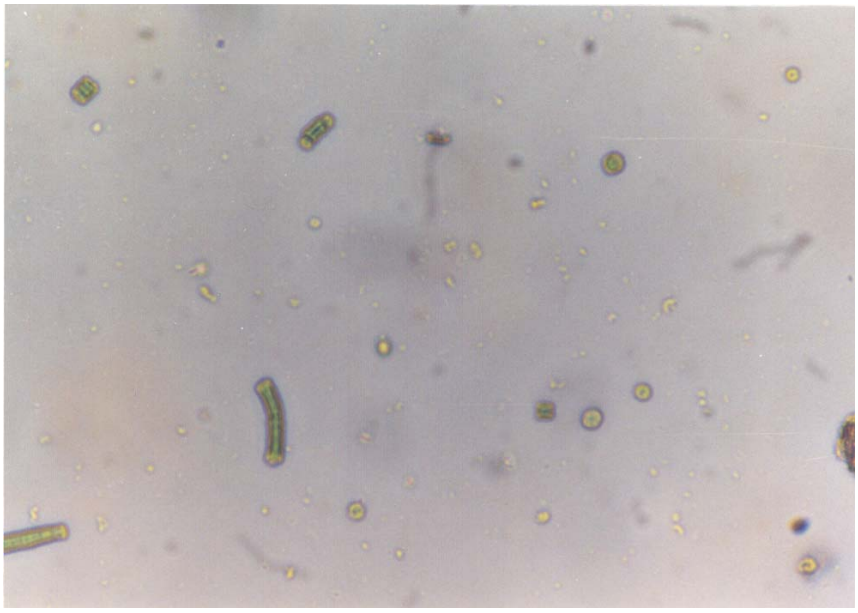
As already indicated, the protoplasts obtained by the first method were not found to be regenerable. The protoplasts prepared by the latter method could be regenerated back to trichomes. The regeneration was monitored by microscopic observation. **Figures 10 A &: B** show the trichomes at two different stages of regeneration. **Figure 10C** gives the picture of completely grown filaments. The regeneration efficiency obtained by different treatments was determined after incubation in three different regeneration media. These results are presented in **Table 2**. Although the extent of regeneration did not have a regular trend, it appeared to be better for younger cultures than for older cultures. Normal Zarrouk's medium was found to be the best medium for regeneration. The maximum regeneration efficiency achieved was 65. The efficiency was expressed as the number of regenerated trichomes per 100 viable cells. **Figure 10D** shows the algal growth regenerated from the protoplasts plated. The trichome regeneration observed in our experiments was due to true protoplasts, as there were no trichomes in the protoplast suspension before they were transferred to regeneration medium.

In conclusion, these experiments demonstrate for the first time that it is possible to obtain viable, regenerable protoplasts from various stages of a growing culture of S.platensis. Further, the efficiency of regeneration can vary drastically depending upon the conditions under which the protoplasts are obtained and subsequently treated. The partial or complete removal of the external sheath by exposure to a high concentration of NaCI was found to be essential for the formation of protoplasts. Due to this pretreatment of the trichomes, the concentration of lysozyme required for cell wall digestion could be kept minimum (0.0196) thus avoiding cellular damage and injury.

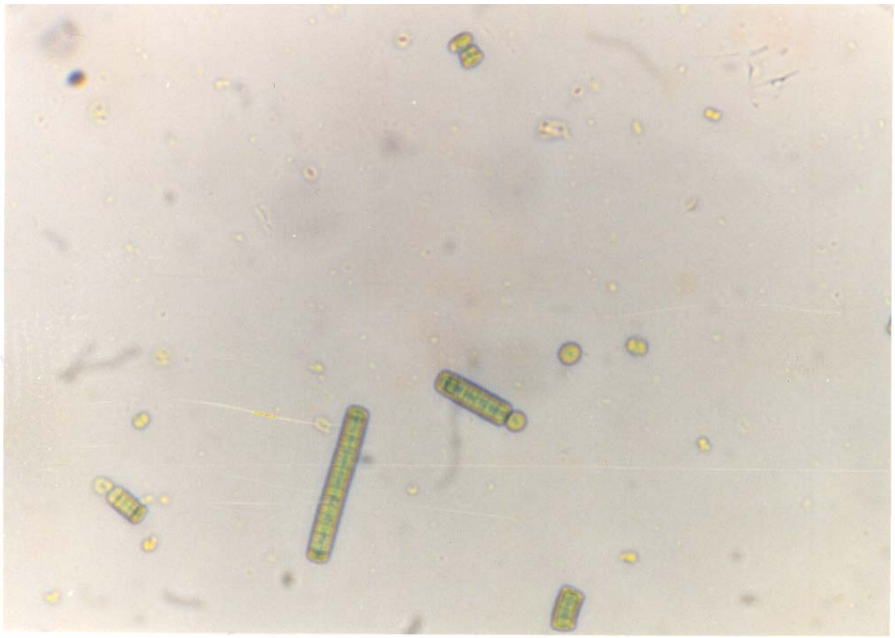
Figure 10. Regeneration of the prepared protoplasts

(Method 2; Culture stage – 4; A_{560} – 0.55; magnification: 670x)

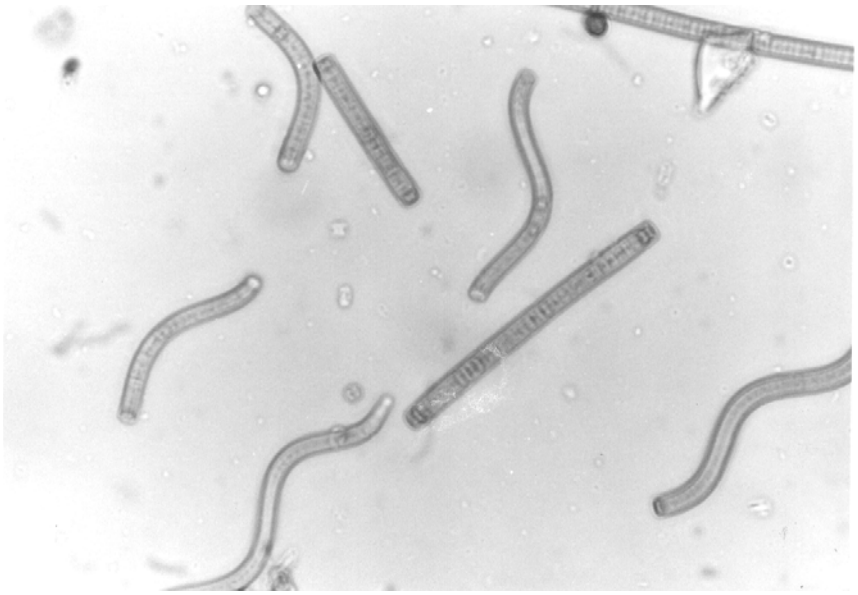
A) Growing filaments at different stages of regeneration (4^{th} day in the regeneration medium)



B) Regenerating trichomes (7 th day in the regeneration medium)



C) Completely regenerated filaments (10^{th} day)



D) Algal growth regenerated from plated protoplasts (after 3 weeks)

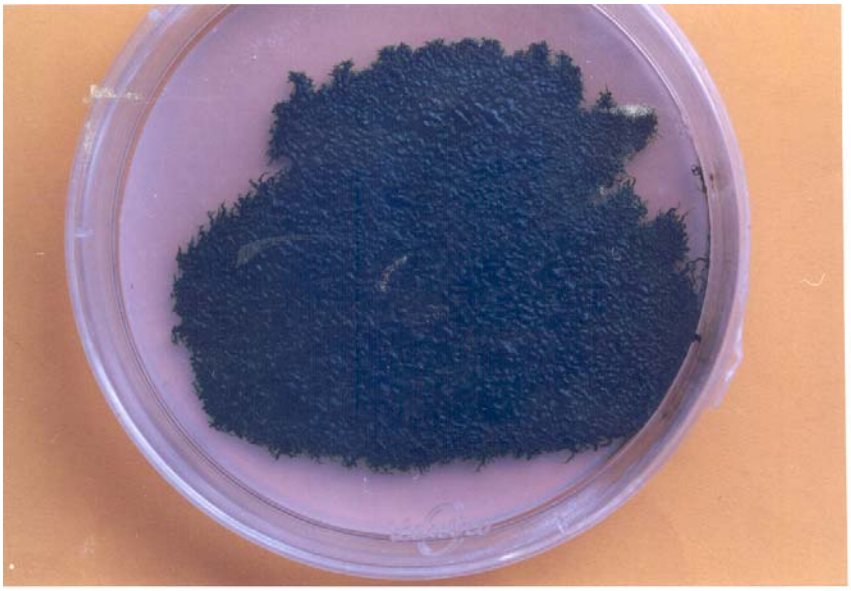


Table 2: Regeneration of Spirulina protoplasts to filaments/trichomes

Stage of culture used for protoplast preparation	A_{560} (corresponding to the stage)	ZM I	ZM II	ZM III
		No. of regenerated trichomes*		
1	0.23	62	59	62
2	0.39	65	64	60
3	0.48	42	40	33
4	0.55	25	24	1 1
5	0.61	19	19	12
6	0.89	16	16	15
7	1 . 1 1	24	16	18
8	1 .53	9	8	8

The regeneration media were i) ZM I-Zarrouk's medium

ii) ZM II -ZM + 1 roM MgC12

iii) ZM III -ZM + 0.05% glucose

The values given above are averages of three independent experiments.

* Regeneration efficiency is expressed as number of trichomes regenerated from a representative population of 100 protoplasts (Regeneration was monitored by counting the no. of trichomes in the regeneration medium after 7 days)

Note: The number of trichomes was zero in the freshly prepared protoplasts suspension before transferring to the regeneration medium.

Experiments on Transformation of Spirulina

No mechanism has been developed or reported so far for the genetic recombination of Spirulina because of 3 major inherent problems in this organism.

1) Lack of plasmids and inability to harbor exogenous plasmids

2) Non-colony forming property

3) Filamentous nature

Keeping this in view, some preliminary attempts were made here to check the ability of Spirulina cells for direct plasmid uptake. Both trichomes and protoplasts were used for the same.

Tables 3 &. 4 give the sensitivity levels of Spirulina cells to two different antibiotics, ampicillin and tetracycline. Since the plasmid pBR322 codes for both ampicillin and tetracycline resistance, the extent of sensitivity of Spirulina cells was determined initially for future selection of transformants based on the antibiotic resistance.

At 25 and 50 μg/ml of ampicillin levels, the alga grew, though not comparable with the control cells. At 75 μg/ml, the algal growth was completely inhibited and at 100 yg/ml and above, the cells were found to be dead. Similarly in the case of tetracycline, there was some at 10 and 20 μg/ml levels, which was retarded at 30 μg/ml and at 40 μg/ml, the cells died completely. Thus, the cells showed 100% sensitivity at a concentration of 100 μg/ml for ampicillin and 40 μg/ml for tetracycline. These levels were used for the transformation studies thereafter.

The uptake of pBR322 by the intact trichomes (made competent) and by freshly prepared protoplasts of S.platensis was studied as a function of period of contact with the donor DNA. In the earlier reports on transformation of cyanobacteria with derivatives of their endogenous plasm ids, transformants were observed after contact periods of <.1 hr [8]. In contrast to these, Spirulina required longer incubation with the plasmid DNA (9 hrs).

Table 3 : Determination of the sensitivity levels of Spirulina trichomes for ampicillin

	AS60 of the culture					
	Test Amp+			Contrl Amp-		
Ampicillin conc (μg/ml)	Initial O. D.	O.D. after 1 day	O. D. after 2 days	Initial O. D.	O. D. After 1 day	O. D. after 2 days
25	0.348 (.04)	0.408 (.03)	0.501 (.06)	0.348 (.04)	0.497 (.05)	0.728 (.07)
50	0.345 (.04)	0.387 (.05)	0.485 (.05)	0.345 (.04)	0.483 (.06)	0.715 (.08)
75	0.349 (.05)	0.351 (.03)	0.362 (.06)	0.349 (.05)	0.501 (.05)	0.741 (.06)
100	0.351 (.03)	cell death	cell death	0.351 i.03)	0.499 (.05)	0.745 (.04)
125	0.342 (.04)	cell death	cell death	0.342 (.04)	0.475 (.04)	0.708 (.06)
150	0.346 (.02)	cell death	cell death	0.346 (.02)	0.498 (.06)	0.740 (.07)

Control -Cell growth in normal growth medium

Test-Cell growth in medium containing ampicillin

The experiments were conducted in triplicates and the standard deviation is given in brackets

Cell death indicates 100% death of cells

Cell death -Visualized, by bleaching of colour and confirmed by viability test described earlier

Table 4 : Determination of the sensitivity levels of Spirulina trichomes for tetracycline

	A_{560} of the culture					
Test Tet+				Control Tet-		
Tetracycline conc.(μg/ml)	Initial O. D.	O. D. after 1 day	O.D. after 2 days	Initial O.D.	O.D. after 1 day	O.D. after 2 days
10	0.321	0.461	0.619	0.321	0.456	0.623
	(.04)	(.05)	(.04)	(.04)	(.05)	(.07)
20	0.326	0.394	0.505	0.326	0.461	0.638
	(.05)	(.03)	(.06)	(.05)	(.05)	(.04)
30	0.318	0.339	0.351	0.318	0.450	0.612
	(.03)	(.06)	(.04)	(.03)	(.05)	(.07)
40	0.323	cell	cell	0.323	0.458	0.631
	(.02)	death	death	(.02)	(.06)	(.06)
50	0.315	cell	cell	0.315	0.449	0.601
	(.03)	death	death	(.03)	(.07)	(.08)

Control -Cell growth in normal growth medium

Test-Cell growth in medium containing tetracycline

The experiments were conducted in triplicates and the standard deviation is given in brackets

Cell death indicates 100% death of cells

Cell Death -Visualized by bleaching of colour and confirmed by viability test described earlier

The plasmid pBR322 (4.3 Kbp), is an extremely versatile CoIE1 type cloning vector with two antibiotic resistant markers, and the complete nucleotide sequence of the plasmid is also known. The genetic map of this plasmid is shown in page 102. Using a similar approach, successful transformation by pBR322 has been reported in Synechococcus 6301 [9, 10] with either intact cells or highly permeable cells called permeaplasts. Though Spirulina lacks plasmids, it is possible that hybrid bacterial/cyanobacterial plasmids developed for other cyanobacteria may also be effective for transforming Spirulina [1].

Checking for transformants (in the intact trichomes)

Figure 11 A shows an untreated intact trichome. The trichomes after KCl wash were treated with MOPS-CaCI2 and made competent. The competent cells were incubated with plasmid DNA in dark for 9 hrs at 40C. These cells were grown in normal growth medium for 24 hrs after which they were transferred to selective broth containing ampicillin (100 μg/ml or tetracycline (40 μg/ml).

The microscopic picture of these trichomes after two days in ampicillin containing medium indicated survival of very few cells, which could probably be the transformants (Fig. 11B). The control cells treated similarly but not incubated with the plasmid resulted in 100% death of the cells in the selective broth. Figure 11C indicates the formation of filaments from the surviving cells (shown above in Fig. 11B) in 7 days. The filamentous morphology of the trichome treated with the plasmid pBR322, after 7 days of growth in ampicillin containing selective medium is shown in Figure 11C.

It is known that the transformants, due to their β-lactamase activity) rapidly degrade ampicillin in the medium [9]. As checked by the bioassay experiments using ampicillin sensitive E.coli HB10l strain, there was a loss in the selection pressure. This probably resulted in the formation of Spirulina filaments with a few untransformed cells in between (Fig. 11 C). This observation was confirmed by transferring such filaments into fresh selective ampicillin containing medium. Microscopic observations indicated

pBR322

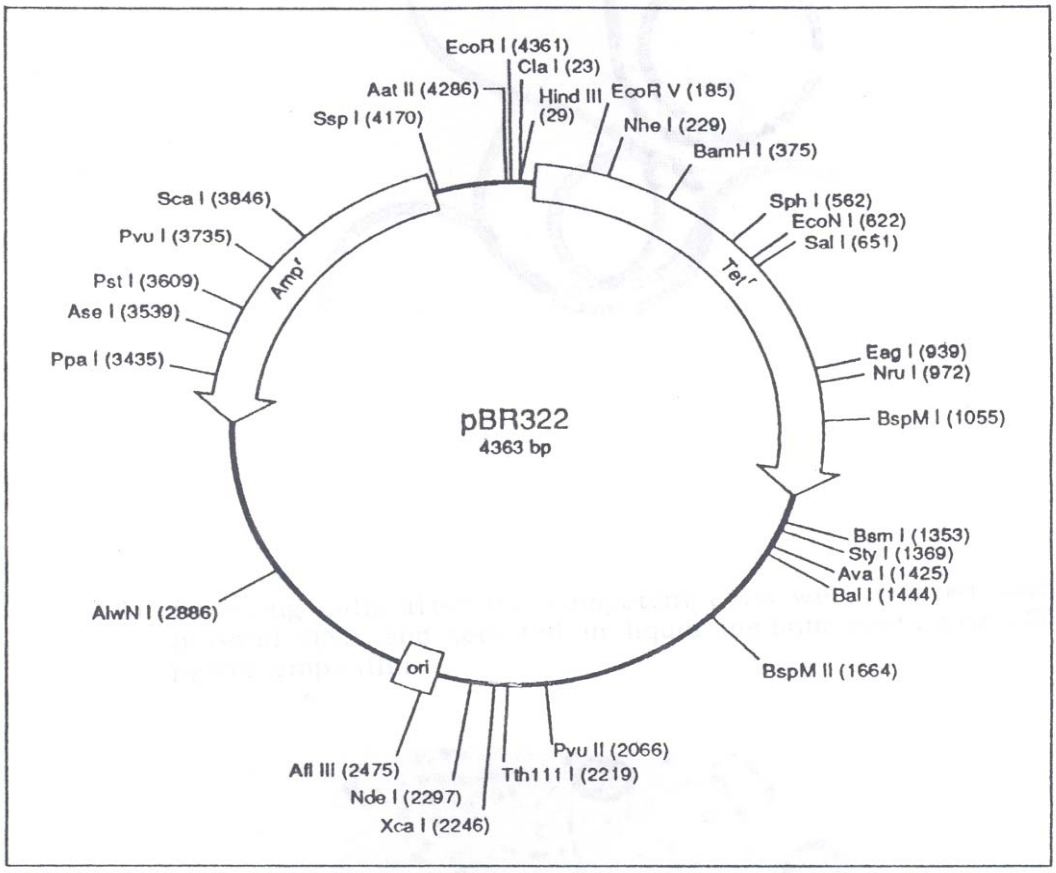
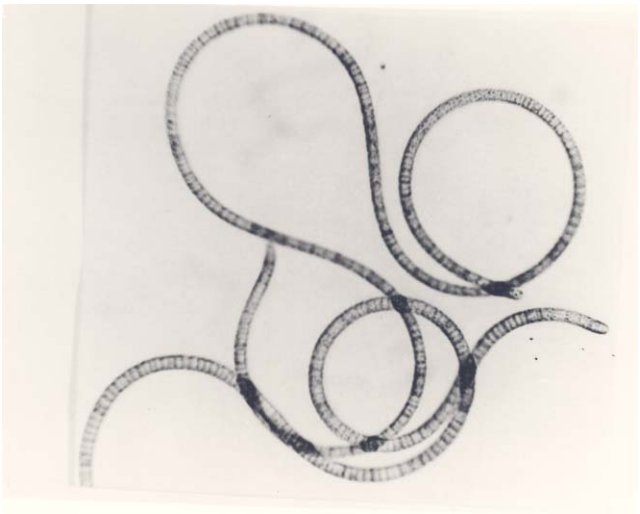
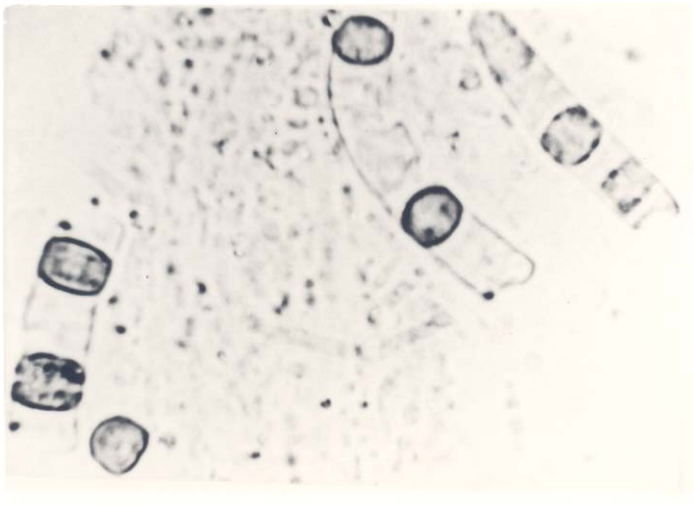

pBR322
4363 bp
EcoR I (4361)
Cla I (23)
Aat II (4286)
Hind III (29)
EcoR V (185)
Ssp I (4170)
Nhe I (229)
BamH I (375)
Sca I (3846)
Sph I (562)
EcoN I (622)
Pvu I (3735)
Sal I (651)
Pst I (3609)
Amp'
Tet'
Ase I (3539)
Ppa I (3435)
Eag I (939)
Nru I (972)
BspM I (1055)
Bsm I (1353)
Sty I (1369)
Ava I (1425)
Bal I (1444)
AlwN I (2886)
ori
BspM II (1664)
Pvu II (2066)
Afl III (2475)
Tth111 I (2219)
Nde I (2297)
Xca I (2246)

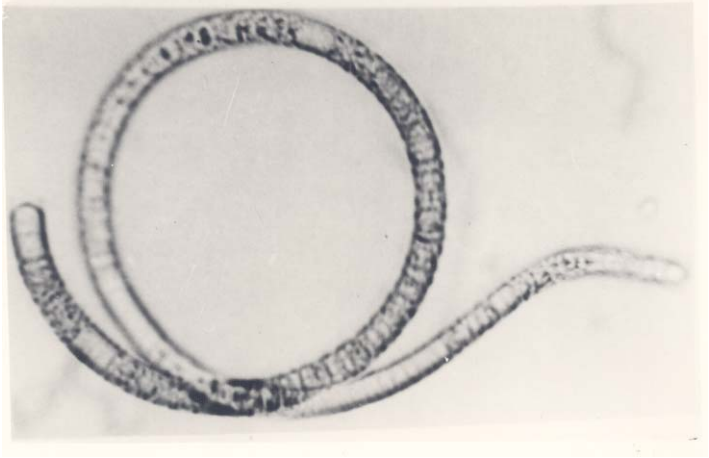
Figure 11. Microscopic obser va t ions at d if f er en t stages dur ing transformation studies with intact trichomes of S.platensis (based on cell resistance to 100μg/ml ampicillin)A) Trichomes &rowing in non-selective liquid medium (Normal growth medium)



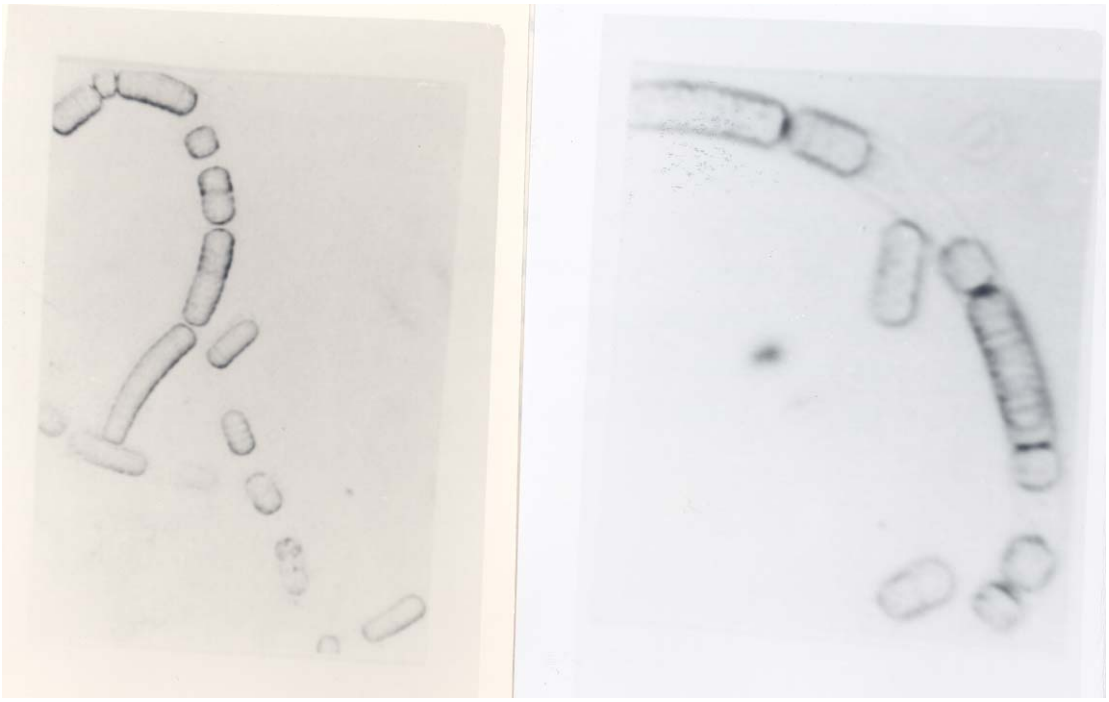
B) Surviving cells after the competent cells were treated with plasmid DNA and selected in liquid medium containing 100 μg/ml ampicillin



C) Filamentous morphology of the trichome after the plasmid uptake (after 7 days of growth in ampicillin containing selective medium)



D) Intracellular lysis of transformed filaments when the filaments were inoculated into fresh selective medium after 7 days of growth



lysis of some cells in the trichome (probably they are non-transformants) due to ampicillin activity (Fig. lID). The ampicillin was added periodically to the selective medium at 100 pg/ml, whenever the selection pressure was lost

Tables 5 & 6 show the resistance level of the test cells and the control cells to antibiotics. The transformation were monitored by their resistance to 100 μg/ml ampicillin **(Table 5)** or 40 μg/ml tetracycline (Table 6). The competent cells incubated with the plasmid DNA showed ampicillin/tetracycline resistance, whereas, the control competent cells which were not incubated with the plasmid DNA were 100% sensitive to the level of antibiotic used.

The exact transformation frequency could not be obtained due to the filamentous nature of Spirulina.After 2 days incubation in selective broth, it could be assumed that nearly 0.12% of transformants arise after treatment of the trichomes with the plasmid DNA, based on the standard O.D. of the culture at 560 nm and cell count by microscopy.

Checking for transformants (in the prepared protoplasts)

In the case of transformation studies using protoplasts, the protoplasts after incubation with the plasmid DNA were plated in the normal growth medium for regeneration and grown for 1 week. After 1 week, the algal growth was transferred to selective liquid medium containing either ampicillin or tetracycline. The extent of sensitivity of the test and the control cells to ampicillin and tetracyline individually is presented in Tables 7 & 8, respectively. As indicated earlier, the exact transformation efficiency could not be determined. It could be assumed from the cell count and A560, that about 0..55% of the transformants arise after treatment of the protoplasts with the plasmid DNA. The frequency of transformation was thus found to be more for protoplasts compared to trichomes. This indicates that the protoplasts are more suitable for direct DNA uptake than trichomes. It requires further experimentation to establish this.

Table 5 : Transformation studies with intact trichomes of Spirulina (based on cell resistance to 100 μg/ml ampicillin)

Days of growth	TEST		CONTROL	
	Competent cells incubated with plasmid DNA		Competent cells not incubated with plasmid DNA	
	Amp+	Amp-	Amp+	Amp-
	A_{560}		A_{560}	
Immediately after transfer	0.435	0.428	0.439	0.436
	(.05)	(.06)	(.06)	(.04)
After 1 day	0.1% (viable cells counted)	0.49 (.04)	100% Death of cells	0.47 (.05)
After 2 days	0.12% (viable cells counted)	0.50 (.06)	No visible cells	0.52 (.07)
After 4 days	Visible cells growing into filaments	0.55 (.04)	No visible cells	0.52 (.05)
After 6 days	~51-54 Trichomes/10μl counter	0.60 (.05)	No visible cells	0.61 (0.08)

* A_{560} -0.44 corresponds to approximately 4.4 x 106 trichomes/ml The experiments were conducted in triplicates and the standard deviation is given in brackets.

Cell death -Visualized by bleaching of colour and confirmed by viability test described above.

The viable cells and the growing filaments were counted in the Burker chamber under a phase contrast light microscope.

Table 6 : Transforaation studies with intact trichomes of Spirulina (based on cell resistance to 40 pg/ml tetracycline)

Days of growth	TEST		CONTROL	
	Competent cells incubated with plasmid DNA		Competent cells not incubated with plasmid DNA	
	Tet+	Tet-	Tet+	Tet-
	A_{560}		A_{560}	
Immediately after transfer	0.415 (.04)	0.417 (.06)	0.412 (.04)	0.419 (.04)
After 1 day	0.1% (viable cells counted)	0.452 (.05)	100% death of cells	0.465 (.06)
After 2 days	0.11% (viable cells counted)	0.471 (.06)	No visible cells	0.478 (.08)
After 4 days	Visible cells growing into filaments	0.514 (.03)	No visible cells	0.523 (.06)
After 6 days	-45-47 Trichomes/10µl counter	0.565 (.04)	No visible cells	0.570 (0.07)

* A_{560} -0.42 corresponds to approximately 4.2 x 10b trichomes/ml The experiments were conducted in triplicates and the standard deviation is given in brackets.

Cell death -Visualized by bleaching of colour and confirmed by viability test described above.

The viable cells and the growing filaments were counted in the

Burker chamber under a phase contrast light microscope.

Table 7 : Transformation studies with Spirul!na protoplasts (based on cell resistance to 100 pg/ml ampicillin)

TEST Protoplasts incubated with plasmid DNA \		CONTROL Protoplasts not incubated with plasmid DNA /	
Plated on normal growth medium (grown for 1 week) |			
Cells transferred to selective broth			
Days of growth	Competent cells incubated with plasmid DNA		Competent cells not incubated with plasmid DNA	
	Amp+	Amp-	Amp+	Amp-
	A_{560}		A_{560}	
Immediately after transfer	0.389 (.03)	0.381 (.03)	0.371 (.02)	0.376 (.04)
After 1 day	0.1% (viable cells counted)	0.399 (.05)	100% death of cells	0.401 (.09)
After 2 days	0.5% (viable cells counted)	0.425 (.06)	No visible cells	0.438 (.07)
After 4 days	Visible cells growing into filaments	0.456 (.06)	No visible cells	0.467 (.05)
After 6 days	~205-220 Trichomes/10µl counter	0.211 (.04)	No visible cells	0.492 (0.06)

* A_{560} -0.39 corresponds to approximately 3.9 x 106 trichomes/ml The experiments were conducted in triplicates and the standard deviation is given in brackets. .

Cell death -Visualized by bleaching of colour and confirmed by viability test described above.

The viable cells and the growing filaments were counted in the Burker chamber under a phase contrast light microscope.

Table 8 : Transformation studies with Spiru!ina protoplasts (based on cell resistance to 40 μg/ml tetracycline)

TEST Protoplasts incubated with plasmid DNA \		CONTROL Protoplasts not incubated with plasmid DNA /	
Plated on normal growth medium (grown for 1 week) |			
Cells transferred to selective broth			
Days of growth	Competent cells incubated with plasmid DNA		Competent cells not incubated with plasmid DNA	
	Tet+	Tet -	Tet +	Tet -
	A_{560}		A_{560}	
Immediately after transfer	0.343 (.02)	0.345 (.03)	0.339 (.02)	0.352 (.05)
After 1 day	0.48% (viable cells counted)	0.359 (.07)	100% death of cells	0.361 (.08)
After 2 days	0.52% (viable cells counted)	0.381 (.04)	No visible Cells	0.384 (.06)
After 4 days	Visible cells growing into filaments	0.412 (.03)	No visible cells	0.430 (.09)
After 6 days	~175-190 Trichomes/10μl counter	0.448 (.05)	No visible cells	0.463 (0.07)

* A_{560} -0.34 corresponds to approximately 3.4 x 106 trichomes/ml The experiments were coQducted in triplicates and the standard deviation is given in brackets.

Cell death -Visualized by bleaching of colour and confirmed by viability test described above.

The viable cells and the growil1g filaments were counted in the Burker chamber under a phase contrast light microscope.

Plating of transformants on LB broth indicated no bacterial contamination. Prolonged contact of donor DNA with recipient cells is also essential in the transformation of S.platensis as shown for Anacystis nidulans [9].

Restriction enzymes Ava I and Ava II present in cyanobacteria were found to reduce the retention

of DNAs containing sequences for these sites [11]. Absence of these restriction sites and presence of fewer restriction sites in the plasmid pBR322 [12], used in the study probably indicate that Spirulina is transformable by exogenous plasm ids like pBR322.

The studies conducted here are preliminary and it may be too early to comment on the Spirulina transformants. More extensive studies are required to establish genetic transformation in this organism. The stability of the transformants in the successive generations needs further analysis. The poor transformation efficiency observed here by using direct DNA uptake upon simple incubation with the plasmid may be perhaps improved by using more effective DNA uptake methods like electroporation or particle bombardment. Whether the plasmid is autonomous or integrated with the chromosomal DNA needs to be elucidated.

In the cyanobacterial classification system, heterocystous nitrogen fixing forms are considered evolutionarily more advanced than the nonheterocystous forms like Spirulina.Conjugal transformation of Nostoc, Anabaena and Anacystis describe use of cyanobacterial replicons like pDU I and pSG III [11, 13] for plasmid replication in cyanobacteria.Forms like Spirulina appear closer to gram negative bacteria in the evolutionary ladder. This is further supported by the identification of a close functional similarity in the elongation factor Tu between S.platensis and E:'~ [14] and detection of the genes in Spirulina using E.coli probes [15-17].

Genetic techniques for the study of photosynthesis, nitrogen fixation and cell differentiation in filamentous cyanobacteria have developed slowly. There is no reproducible transformation procedure for filamentous cyanobacteria as there is for several strains of unicellular cyanobacteria [18, 19]. The only well-documented method for gene transfer in filamentous cyanobacteria is conjugative transfer of shuttle vectors from Escherichia coli by using a broad-host range plasmid such as RP4 [11,20-22].

Previous reports of cyanobacterial transformation have involved the use of modified endogenous plasm ids [23-27], chimeric plasmids constructed from cyanobacterial and E.coli plasmids [13, 28-32], or chimeric DNA molecules consisting of cyanobacterial chromosomal DNA linked to antibiotic resistance genes from E.coli [33, 34]. In all these cases, homology between the chromosome and plasmid facilitated transformation, although the precise mechanism of integration of plasmid is not elucidated. In other organisms, chromosomal integration of plasmid DNA has been accomplished by constructing replicon-defective plasm ids carrying a segment of DNA homologous to the chromosome of the recipient organisms [35-37]. Additionally, homology between plasmid and host chromosome DNA greatly enhances transformation [38]. Exchange of genes between plasmid and host chromosome during transformation has also been well documented [38]. In a related study, it has been found that, when a plasmid is established via homologous recombination with the chromosome, plasm ids isolated from transformants may be of the

same size as the plasmid used for the initial transformation. Moreover, the reisolated plasmids contain segments of the host chromosome [39].

It should be noted that genes of interest in photosynthesis or nitrogen fixation have been cloned in pBR322 or its derivatives, but not as yet in any of the cyanobacterial shuttle vectors. Regardless of the mechanism, establishment, stability and replication of the plasmid, preliminary attempts on transformation of Spirulina with the versatile cloning vector pBR322 opens a new possibility for genetic manipulation of this organism. The significant contribution in this chapter is the development of regenerable protoplasts which may serve as efficient tools for transformation studies. The study also shows a promise for transformation of Spirulina by a pBR322-like plasmid.

EXPERIMENTAL

Chemicals used

Lysozyme and fluorescein diacetate were obtained from Sigma Chemical & Co. pBR322 plasmid was purchased from Bangalore Genei Pvt. Ltd. All the other chemicals used were of the highest quality available locally. The water used throughout was triple distilled, deionized and microfiltered.

Formation, purification and regeneration of protoplasts

Axenic Spirulina platensis (FTCC 06) available in our germplasm collection was grown in Zarrouk's medium [40]. The growth curve of a typical culture isshown in Figure 1.At the indicated time points, different volumes of the culture were withdrawn and centrifuged at 10,000 x g for 10-20 min to yield approximately the same amount of trichomes (- 4x 106) from each sample, as monitored by A560'

All the manipulations described below, with the exception of the microscopic observations, were carried out under sterile conditions.

Protoplast preparation and purification (Method 1)

The trichomes were washed twice in 0.5 M mannitol, 0.03 M potassium phosphate buffer (pH 6.8). They were suspended in the mannitol-phosphate buffer with 2 mM EDT A (at a density of 4xl04 trichomes/ml) and lysozyme was added to give a final enzyme concentration of 0.1% (w/v). The cells were incubated at room temperature for 28 hrs with occasional stirring (at 30 rpm) and then cooled in an ice bath. The protoplasts formed were separated from the cell debris on a sucrose density gradient by the floatation method and collected by centrifugation at 500 x g for 4 min. They were resuspended in fresh

mannitol phosphate and resedimented to remove excess lysozyme [6].

Protoplast preparation and purification (Method 2)

The trichomes were resuspended in 1 ml of Zarrouk's medium supplemented with 1.5 M NaCl. This was centrifuged (3,000xg, 1 min) and the cells suspended in Solution (Sol. P; pH 8.0) of 10 mM Tricine-NaOH, 2mM MgC12, 2mM CaC12, 40mM NaCl and 10% PEG-4000. Centrifuged (3,000xg, 1 min) to yield pellet which was resuspended in 1 ml Sol. P. This sample was divided into two portions and centrifuged (3,000xg, 1 min) to yield pellets which were resuspended in 1 ml of i) Sol. P with lysozyme (0.010;0) and ii) Sol. P without lysozyme. The samples were incubated at 350C with occasional stirring. Small aliquots were removed at 10-15 min intervals for observation under the light microscope; the treatment was stopped by low speed « 50 x g) centrifugation when the majority of the trichomes appeared to have given rise to protoplasts and possibly to a fe\v single cells (after 60-70 min) (Fig 2).

The resulting pellets were then suspended in 100 µ1 Sol. P, layered onto a discontinuous albumin gradients of 2 ml layers each of albumin at 2.5, 5.0, 7.5 and 10.096 in Sol. P.Centrifuged at 50 x g for 10-45 min. Protoplasts and single cells, which formed a band at the top were collected and their number and purity were checked by observation under the microscope in a Burker chamber (9 mm2 fields were checked for the preparation).

Viability of the protoplasts

A sample of the protoplast suspension was stained by mixing with an equal volume of 2.5% Evans blue in Sol. P. The viable protoplasts or cells appeared light green in colour while damaged or non-viable cells appeared dark blue in colour due to dye-inclusion.

Stock solutions for the viability assay involving two dyes were prepared by dissolving fluorescein diacetate (FDA) in acetone (5 mg/ml) and calcofluor white in growth medium (2.5 mg/ml) and stored at Ooc. Aliquots of the assay stock solutions were added to the protoplast suspension to a final concentration of 0.0190 FDA and 0.02596 calcofluor white. The stained cells

were incubated for 5 min at room temperature and their fluorescence determined using a using a Leitz Diaplan fluorescent microscope with an exciter filter passing light between 450-490 nm.

Later the method was modified involving only calcofluor white dissolved in growth media (2.5 mg ml-I). This was added to the cell suspension to a final concentration of 0.025% calcofluor white. The stainedcells were incubated for 5 min at room temperature and their fluorescence determined. The viable cells fluoresced red in colour whereas the non-viable cells fluoresced blue in colour (Fig. 3). Viability was

calculated as the ratio of viable/non-viable cells after counting 400 cells.

Regeneration of the protoplasts

To test for regeneration, 0.15 ml aliquots of the protoplast preparation were mixed with 2.85 ml of the desired regeneration medium (see legend to Table 1) and incubated for 2 days at low light intensity and then for 4 days under standard conditions of lighting and temperature. To determine the extent of regeneration, the cells were pelleted by centrifugation (10,000 x g, 30 min), resuspended in 0.15 ml of the corresponding regeneration medium and counted in the Burker chamber as described above. The percentage regeneration was expressed as the number of regenerated trichomes from a representative population of 100 protoplasts. There were no trichomes present in the protoplast suspension before regeneration.

Transformation work on Spirulina

Transformation studies with intact trichomes

Cells from a young actively growing axenic culture (0.5 O.D. at 560 nm) of Spirulina platensis (FTCC 06) were pelleted by centrifugation at 4oC (10,000 x g for 15 min). The cells were treated with 10 ml of 2M NaCI for 10 min at 4oc to remove the sheath and washed in 0.1 M M0PS (pH 6.5) containing 60 mM CaCl2 at 4^0C, followed by centrifugation (5,000 x g, 15 min) at 4^oC. The pelleted cells were made competent by suspending them in 1.5 ml of the same MOPS-CaCI2 mixture.

250µ1 of the competent cells treated to 2.0 µg plasmid DNA (pBR322) was incubated at 40C in the dark for transformation with gentle shaking (30 rpm). Aliquots were removed after 3, 6, 9, 14' and 18 hrs from the incubation mixture. Following heat shock at 42^0C for 10 min, the cell suspension inoculated into 10 ml of the medium was grown for 24 hr on a rotary platform shaker under light (90 rpm, 100µE m-2 s-1). Since the plasmid pBR322 codes for both ampicillin and tetracycline resistance, the transformants were selected by growth in liquid medium containing 100 µg/ml ampicillin or 40 µg/ml tetracycline. Ampicillin degradation in the medium was continuously monitored by ampicillin sensitivity assay using plates containing E.coli HB101. Ampicillin at 100 µg/ml levels were supplemented into the medium whenever bioassay indicated loss of ampicillin activity. The transformants were also maintained in liquid medium containing ampicillin constantly.

Transformation studies with prepared protoplasts

Protoplasts prepared by 1 hr treatment with Sol. P (Method 2) were used for transformation immediately. To 1.0 ml of protoplasts, 1 μg of donor pBR322 DNA (in 10 mM Tris, 1 mM EDT A, pH 8.0 at 250c) was added, and the suspension was incubated for different durations in sterile culture tubes in dark at 40c at 30 rpm. Samples were plated in triplicate with a series of 2fold serial dilutions to quantify transformants.

Transformants were selected by incubation of the inoculated plates in the absence of antibiotic at 320C for 1 week for trichome regeneration. Then the algal growth transformed to selective broth containing the antibiotic (100 μg/ml ampicillin or 40 μg/ml tetracycline).

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HIGHLIGHTS OF THE WORK

The following are the highlights of the present work:

* The germplasm collection and screening helped in identifying FTCC 06 as the most suitable strain for commercial exploitation which was found to be the best for biomass, protein and phycocyanin production. FTCC 03 had the highest SOD activity. β-carotene accumulation was more in FTCC 13 than in other strains.
* Eleven principal carotenoids were identified in Spirulina. Among these, neoxanthin, violaxanthin, lutein, phytofluene and phytoene are reported for the first time as the carotenoids of the order Nostacales.
* The 1.5 fold increase in p-carotene obtained by sulphate/nitrate stress and 3 fold increase achieved by UV treatment of cells are significant in terms of the nutritional value of Spirulina.
* The 8, 18 and 27% enhancement in phycocyanin achieved by nitrate stress is economically viable in view of its high market value.
* A rapid method was developed for the isolation of intact phycobilisomes in 30 sec using CTAB followed by purification on sucrose gradient. Proteolysis of phycobiliproteins and chlorophyll contamination were minimal. The structural andfunctional properties were retained as evidenced by spectroscopy and SDS-PAGE.
* SOD has been purified from Spirulina for the first time in view of its applicability as a potential antioxidant and in view of value addition to Spirulina. The enzymological properties were studied. The purified enzyme preparation exhibited an antioxidant effect of 0.94 in oxidation studies with linoleic acid emulsion. The enzyme was also immobilized and studied.
* A method was standardized for the isolation of viable protoplasts, but they were not regenerable. Hence, another rapid method was later developed for protoplast preparation with a good regeneration capacity. The yield was found to be 86% with a regeneration efficiency of 65. A novel viability assay was also developed to differentiate the viable cells from the nonviable cells.
* Attempts were made and preliminary evidence was obtained for the first time for direct transformation of Spirulina (trichomes/protoplasts), by using a shuttle plasmid vector, pBR322. The transformation was monitored and transformants selected based on their resistance to ampicillin or tetracycline.

Development of mass production of Spirulina depends on the evaluation of the performance of the strains in terms of growth velocity, yield, chemical composition, and susceptibility to contamination by other microorganisms when utilized on continuous or semicontinuous processes. On a laboratory scale, it may be especially fruitful to search for natural strains or for the isolation of mutants endowed with more favourable characteristics. Finally, any extensive genetic alteration of the presently available strains

depends on the unravelling of the mechanism for genetic recombination or the development of other means to manipulate Spirulina genetically.

THE FUTURE LEADS

The commercial applications of microalgal culture, especially Spirulina are numerous and ever expanding. They include health foods, production of vitamins and pharmaceuticals, food coloring agents, metal biosorbents and aquaculture feeds. This apart, microalgae show promise in the industrial waste treatment and in the production of fuels, soil inoculants, isotopically labelled compounds and diagnostic reagents. However, production scales are relatively small and only the initial steps have been taken for successful commercialization.

The studies conducted here show immense promise for further research on this organism, clearly indicating the future leads therefrom.

At present, developing micro-algae for commercial use depends on selection, screening and culturing natural species. As a result, advances in mass culture technology, aimed at manipulating environmental conditions to enhance the quantity and quality of the organism will have the largest near-term impact. The selection and screening of natural strains for high yield were carried out in this study which is the most practical way of achieving a substantial increase in productivity.

When the industrial product is not the cell mass but a cellular product (e.g. β-carotene, phycocyanin, GLA, SOD, etc.), the target is to increase the natural content of the desired product in the cell. If, for example, the percentage of β -carotene in Spirulina could be raised by proper selection and mutation, to 8-9% of the dry weight as in Dunaliella bardawil, the economic basis for that industry may be strengthened.

The cellular constituents can also be enhanced by manipulating the nutrients supplied and the growth conditions (e.g. β -carotene in Dunaliella [1]). Using this approach, the content of phycocyanin and β -carotene in Spirulina platensis- (FTCC 06) cells were enhanced in our experiments by manipulating the nutrients and the growth conditions. This is economically viable since phycocyanin, the blue pigment has a high market value and growing importance in various industries. β -carotene increases the nutritional value of Spirulina.

The cyanobacterial extracts containing carotenoids are frequently used as natural colouring material and owing to the interest on the constituents of the extract from Spirulina, the principal carotenoids were identified.

One of the applications of Spirulina in pharmaceutical industry is its ability to quench free radicals in human system due to SOD levels, thereby reducing health risks and retarding ageing process. SOD in Spirulina can also be looked upon as a potential antioxidant in foods and with this in view, the properties of the purified SOD from Spirulina were studied followed by antioxidant studies and immobilization.

Presently, cell fusion techniques are being tested to obtain algae with both autotrophic and heterotrophic properties. Biotechnological Resources (USA) has used mutated algal strains with a shorter doubling time, which can utilize variety of carbohydrates and reach a high productivity of even 100 g/L [2]. No mechanism has been discovered for genetic recombination for Spirulina. If a significant increase in levels is achieved either for GLA, β-carotene, phycocyanin or SOD of Spirulina by cell fusion or genetic manipulation, it will boost the market value and the demand for this alga.

Moreover, due to food and pharmaceutical importance and easy culture conditions, Spirulina could be viewed as an alternate host for heterologous gene expression if genetic recombination methods are developed in this organism. To pave the way for reaching these long term goals, preparation of regenerable protoplasts was standardized and attempts were made to directly transform Spirulina using shuttle vectors. These would lead to future studies on cloning and expression of foreign genes of interest in Spirulina. Protoplasts are also important for fusion studies, for obtaining better genetic recombination of strains.

The preliminary studies on genetic transformation indicate that, Spirulina is transformable by an exogenous plasmid. But, further work needs to be carried out to answer the following questions.

1) Which is the most suitable plasmid for engineering this organism genetically?
2) How stable are Spirulina transformants?
3) Since Spirulina lacks plasmid, can exogenous plasmid taken up by Spirulina cells, establish itself and replicate?
4) Whether the plasmid gets integrated into the chromosomal DNA to give stable transformants or remain autonomous?
5) Can this approach establish that Spirulina can be genetically manipulated for introducing useful traits to obtain improved strains?

These are some of the future leads that the present investigation offers. Taking into consideration the numerous conceivable applications of cyanobacteria and genetic capabilities appropriate to those applications, it is the need of the hour to develop cyanobacterial genetics in an applicationoriented pathway with the help of the powerful tools, genetics has provided to manipulate cyanobacteria of potential biotechnological use.

Micro algal culture, Spirulina in particular, has clearly advanced from a laboratory curiosity to a commercial reality. In addition to the microalgal products already commercialized, a large number of potential products are in various stages of development. Microalgae seem to be the 'wonder gift of nature' and a 'time tested food for the future'. They are said to be an 'indigenous biotech marvel'. Thus, it appears that microalgal mass culture is one of the successes of modern biotechnology and that the future promises rapid expansion and new applications in the field of algal biotechnology.

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